Conservation Genetics of the orange-fronted kākāriki (Cyanoramphus malherbi)

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(*Cyanoramphus malherbi*)

Photo: Julia Melville, Department of Conservation

Contents

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Abstract

The critically endangered orange-fronted kākāriki (*Cyanoramphus malherbi*) is an endemic parakeet restricted to three small breeding populations within the Hawdon, Hurunui and Poulter Valleys of North Canterbury, four translocated populations on offshore islands and a captive breeding facility in Christchurch. Seventeen polymorphic microsatellite loci were developed from next-generation 454-sequencing of genomic DNA. At the commencement of this project, only birds sourced from the Hawdon and Hurunui populations had been used in the captive and translocated populations. To determine appropriate source populations for future translocation, this study used both nuclear and mitochondrial data to quantify the level of genetic diversity within, and the pattern of genetic differentiation among, the three remaining wild populations of *C. malherbi*. Six *C. malherbi* of known provenance from each of the three populations were genotyped at the 17 microsatellite loci and one microsatellite loci previously developed for the closely related Forbe's parakeet (*C. forbesi*), and sequenced at one mitochondrial (cytochrome *b*) and one nuclear exon (RAG-1). For each valley, the number of microsatellite alleles ranged from one to four per locus. Observed and expected heterozygosities ranged from 0.0 to 1.0 and from 0.17 to 0.74, respectively. The Poulter valley had the highest average allelic diversity (2.3) and the highest average observed and expected heterozygosities (0.40 & 0.38, respectively). Weak but significant population genetic structure was detected among valleys (F_{ST} = 0.06, F'_{ST} = 0.09, p = 0.04). Pairwise F_{ST} estimates identified the Hurunui as being significantly different from both the Poulter and Hawdon Valleys (F_{ST} = 0.11, p = 0.01 and 0.061, p < 0.01, respectively). In contrast, STRUCTURE analysis indicated that the three valleys comprise a single genetic cluster. Three cytochrome *b* haplotypes were identified, two of which were found in all three populations and one haplotype that was present in one Poulter valley individual only.

Two RAG-1 alleles were identified, both of which were shared by all three valleys. No significant population genetic structure was detected for either the cytochrome *b* or RAG-1 loci. These combined data suggest that the three North Canterbury valleys function as a metapopulation with some level of connectivity. The same nuclear and mitochondrial markers were used to determine the genetic distinctiveness of sympatric *C. malherbi* and *C. auriceps* North Canterbury populations and identify putative cryptic hybrids from the now extinct Hope Valley population. Based on both cytochrome *b* and microsatellite markers, *C. malherbi* and *C.auriceps* were found to be genetically distinct (ϕ_{ST} = p<0.01; F_{ST} = 0.073, p < 0.01; K=2), and the two Hope Valley birds were confirmed to be hybrids. These findings lend support to the hypothesis that when one species is rare and the other abundant, limited hybridisation between sympatric populations of *C. malherbi* and *C. auriceps* is possible. Overall, this thesis has provided useful genetic tools and information to inform active conservation management of captive and translocated populations of *C. malherbi.* Based on my conclusions, I recommend the inclusion of individuals from each of the three source populations within the captive breeding and translocated populations to conserve current levels of genetic diversity. In addition, to ensure the genetic integrity of *C. malherbi* I strongly recommend the use of these molecular methods to accurately identify all individuals prior to entering the captive breeding and translocation programmes. I also recommend the on-going genetic monitoring and management of captive and translocated populations to guide future conservation management of this critically endangered kākāriki.

Chapter 1: Introduction

Conservation Genetics

Biological conservation encompasses the preservation of ecosystem, species and genetic diversity (Frankham 2010; Sarre & George 2009). The integration of genetics into conservation biology reflects the importance of maintaining genetic diversity and the evolutionary potential across the geographical range of species (Sarre & George 2009). For example, by combining genetic data with ecological, behavioural and morphological data, species, subspecies, and evolutionary significant units can be identified and protected (Frankham 2010; Allendorf & Luikart 2007). Genetic tools have also been used successfully to estimate genetic diversity, population structure, effective population size and gene flow within, between and among threatened populations (Allendorf & Luikart 2007). Furthermore, by combining genetic and demographic data increases our understanding of the historical and contemporary processes that have generated and maintained current spatial structure and genetic diversity (Sarre & George 2009). Funk et al. (2010) for example, used both microsatellite data and long-term demographic data to detect relatively slow rates of population decline in the northern spotted owl (*Stix occidentalis caurina*). Raisin et al (2012) used microsatellite data to demonstrate the successful conservation of genetic diversity during 30 years of intensive conservation management of the critically endangered Mauritius parakeet (*Psittacula echo*). Chan et al (2011) used both mitochondrial and microsatellites to assess the levels of genetic diversity and genetic structure of the Cook Island's endangered flycatcher, the kakerori (*Pomarea dimidiata*). Molecular genetic markers have also been used effectively to determine the levels of hybridisation and introgression between closely related species (Allendorf & Luikart 2007). For example, based on mitochondrial sequence data, microsatellite genotypes and morphological

markers Chan et al. (2006) determined the extent of hybridisation between the Forbes' parakeet (*Cyanoramphus forbesi*) and the Chatham Island red-crowned parakeet (*C. novaezelandiae chathamensis*).

Globally, habitat loss and fragmentation have resulted in both the range contraction and population declines in many endemic bird, amphibian, reptilian and invertebrate species (Jamieson et al. 2009). Oceanic island avifauna is particularly vulnerable to the adverse effects of introduced mammalian predators and habitat modification (Bellingham et al. 2010). After eight hundred years of human inhabitation in New Zealand for example, only 22% of native forest remains (Rhodes et al. 2009) and 41% of endemic birds within the New Zealand archipelago have become locally or globally extinct (Innes et al. 2010). The widespread presence of introduced mammals on islands has well documented negative effects on biodiversity, particularly on isolated islands with high levels of endemism and where native species have evolved in the absence of mammalian predators (Towns et al. 2012; Phillips 2010; Jamieson et al. 2006; Pryde et al. 2005). Introduced predators are implicated in the extinction, substantial population declines or extirpation of many native island species. For example, 75% of all threatened bird species on oceanic islands are a risk from predation from introduced mammals (Birdlife International 2008).

Non-migratory endemic island avifauna with small or declining fragmented and isolated populations are likely to become genetically differentiated from each other and are at risk of inbreeding depression (Raisin et al. 2012; Jamieson et al 2009; Sarre & Georges 2009). Additionally, as a consequence of limited or no gene flow, limited carrying capacity, founder events and low effective populations, endemic island populations generally have lower levels of genetic variation than mainland populations (Miller et al. 2011; Cardoso et al. 2009;

Jamieson et al. 2009, Boessenkool et al. 2007).The resultant loss of fitness and evolutionary potential is expected to increase extinction risk in threatened island species (Frankham 2005). The reduction of effective population size following a severe population bottleneck reduces genetic diversity and increases the probability of inbreeding within a population (Heber & Briskie 2010). In birds, inbreeding is known to reduce hatching success. For example, Heber & Briskie's (2010) global study of 51 threatened bird species found that hatching success decreased by 10% in all populations that passed through bottlenecks of below 100-150 individuals.

The conservation of New Zealand's endangered endemic fauna to date has focused on predator control, habitat restoration, and translocations to predator free off-shore islands (Clout 2001). Over the past decade, however, the protection of genetic diversity and therefore the evolutionary potential of genetically distinct populations have been recognized as essential for successful threatened species management (Jamieson et al. 2006; Robertson 2006; Frankham 2005). Contemporary conservation strategies therefore must continue to protect endangered species from the immediate threats of predation and habitat loss by establishing captive and translocated populations whilst managing the long-term genetic consequences associated with such small and isolated populations (Jamieson et al. 2006; Frankham, 2005). Unfortunately, the size, origin, and genetic diversity of both captive and translocated founder populations are often severely restricted in threatened and endangered species (Witzenberger & Hochkirch 2011).

Conservation genetics is an integral component of conservation management of the following endangered New Zealand birds. The quantification of genetic diversity, molecular sexing and paternity testing for example, has contributed to the conservation management

of New Zealand's critically endangered kakapo (*Strigops habroptilus,* Robertson 2006). Also, Grueber and Jamieson (2008) integrated pedigree data into the genetic management of Takahe (*Porphyrio hochstetteri*) to quantify and manage genetic diversity in free-ranging populations of this critically endangered rail. Steeves et al. (2010) combined mitochondrial DNA sequence data and microsatellite data to assess the extent of hybridisation and introgression between sympatric populations of the critically endangered black stilt (kakī, *Himantopus novaezelandiae*) and the self-introduced pied stilt (poaka, *H. h. leucoephalus).* In addition, Hagen et al. (2011) used microsatellites to study the relationship between genetic relatedness and reproductive success within a small captive breeding population of black stilt. The findings of both studies provided relevant and practical strategies to maintain the genetic integrity and diversity of black stilt (Steeves et al. 2010) and avoid inbreeding depression in captive populations (Hagen et al. 2011)

The Cyanoramphus parakeets

The *Cyanoramphus* parakeets are small to medium sized Psitticine parrots endemic to the South West Pacific, inhabiting islands from New Caledonia to the Auckland Islands (Kearvell et al. 2003; Boon et al. 2000; Taylor 1985; Fig 1). Prior to the availability of genetic tools, determining the phylogeny of this species complex was problematic due to the difficulty in distinguishing between these morphologically similar parakeets (Kearvell et al. 2003). Using mitochondrial control region DNA sequence data, Boon et al. (2000) identified ten *Cyanoramphus* species and four subspecies. Eight of these are found on mainland or off-shore islands of New Zealand (Table1, Fig1).

Table 1: Species of *Cyanoramphus* found on the New Zealand archipelago (Boon et al. 2000)

Common Name	Scientific Name	Distribution
Orange-fronted parakeet	*C. malherbi*	North Canterbury, Chalky Maud, Blumine & Tūhua Islands
Yellow-crowned parakeet	*C. auriceps*	North, South and Stewart & offshore islands
Red-crowned parakeet	*C. novaezelandiae*	Stewart & Auckland Islands & offshore islands
Antipodes Island parakeet	*C. unicolor*	Antipodes Island
Reischek's parakeet	*C. hochstetteri*	Antipodes Island
Forbes' parakeet	*C. forbesi*	Chatham Islands
Chatham Island red-crowned parakeet	*C. n. chathamensis*	Chatham Islands
Kermadec parakeet	*C.n. cyanurus*	Kermadec Islands

Fig1: The New Zealand archipelago

The orange-fronted parakeet (*Cyanoramphus malherbi*) was first described in 1857 (Souancé) but based on the literature and museum specimens Holyoake (1974) concluded that *C. malherbi* was a colour-morph of the yellow-crowned parakeet (*C. auriceps*). This was subsequently supported by a morphological study of museum specimens by Nixon (1981). A review of previous research by Taylor (1998) also concluded that "the two forms are colour-morphs of the same species." However, research into the taxonomic status of *C. malherbi* over the past thirteen years based on morphological (Young & Kearvell 2001), behavioural (Kearvell & Briskie 2003; Boon et al. 2001), ecological (Kearvell et al. 2002), and genetic (Boon et al.2001) analyses indicated that the orange-fronted parakeet was indeed a distinct species. Consequently, conservation management of *C. malherbi* began in 2000 (Kearvell et al. 2003).

Formerly occurring in most of New Zealand, *C. malherbi* has not been observed in large numbers since the late 1800s and has always been considered to be the rarest of New Zealand's *Cyanoramphus* parakeets (Birdlife International 2012; Taylor et al. 1986). The exact historical abundance and distribution of *C. malherbi* remains uncertain as a result of insufficient and possibly inaccurate identification, historical documentation (Triggs & Daugherty 1996; Nixon 1981; Harrison 1970) and the difficulty in accurately distinguishing between *Cyanoramphus* species from fossil remains (Holdaway et al. 2010). Harrison (1970) stated that, "Sight records have been regarded with suspicion since the differences which distinguish this species from its nearest congener, the Yellow-crowned Parakeet, can be discerned only at very close range and in good light." Accurate current population estimates are also hampered by the cryptic and secretive nature of *C. malherbi* and the difficulty in

distinguishing between sympatric *C. malherbi* and *C. auriceps* dwelling in the forest canopy (Ortiz-Catedral & Brunton 2009).

Currently, there are three small breeding populations restricted to North Canterbury beech *(Nothofagus*) forests of the South Branch of the Hurunui within Lake Sumner Forest Park and, the Hawdon and Poulter valleys within Arthur's Pass National Park (Grant & Kearvell, 2001; Fig 2). No nests have been sighted in the Hurunui in the last four breeding seasons (Kearvell pers. comm). During the 1999/2000 breeding season the Hurunui populations experienced a substantial population decline in response to a rat irruption following two consecutive beech mast years (Bird Life International 2012). It is estimated that the total number of *C.malherbi* fell from approximately 500 - 700 birds to around 200 birds (Birdlife International 2012). Captive breeding of *C.malherbi* began in 2003 at Isaacs Wildlife Trust, Peacock Springs (Christchurch). Since 2005, captive-reared offspring from birds sourced from the Hurunui and Hawdon valleys have been translocated to predator-free Maud Island in the Marlborough Sounds, Chalky Island in Fiordland and Tūhua in the Bay of Plenty (Birdlife International 2012, *C. malherbi*). The Poulter Valley population was discovered in 2003 and eggs from this valley were collected for captive breeding in 2009 (*C. malherbi* studbook). The translocated populations have had mixed results. A steady decline in *C. malherbi* and an increase in *C. auriceps* numbers have been observed on Chalky Island and a gradual decline in the *C. malherbi* population has been observed on Maud Island (Kearvell pers. comm.). Translocations to Blumine Island and Tūhua began in 2009 and 2011 respectively. Monitoring of these populations suggests *C. malherbi* are successfully breeding on these islands (Kearvell pers. comm.).

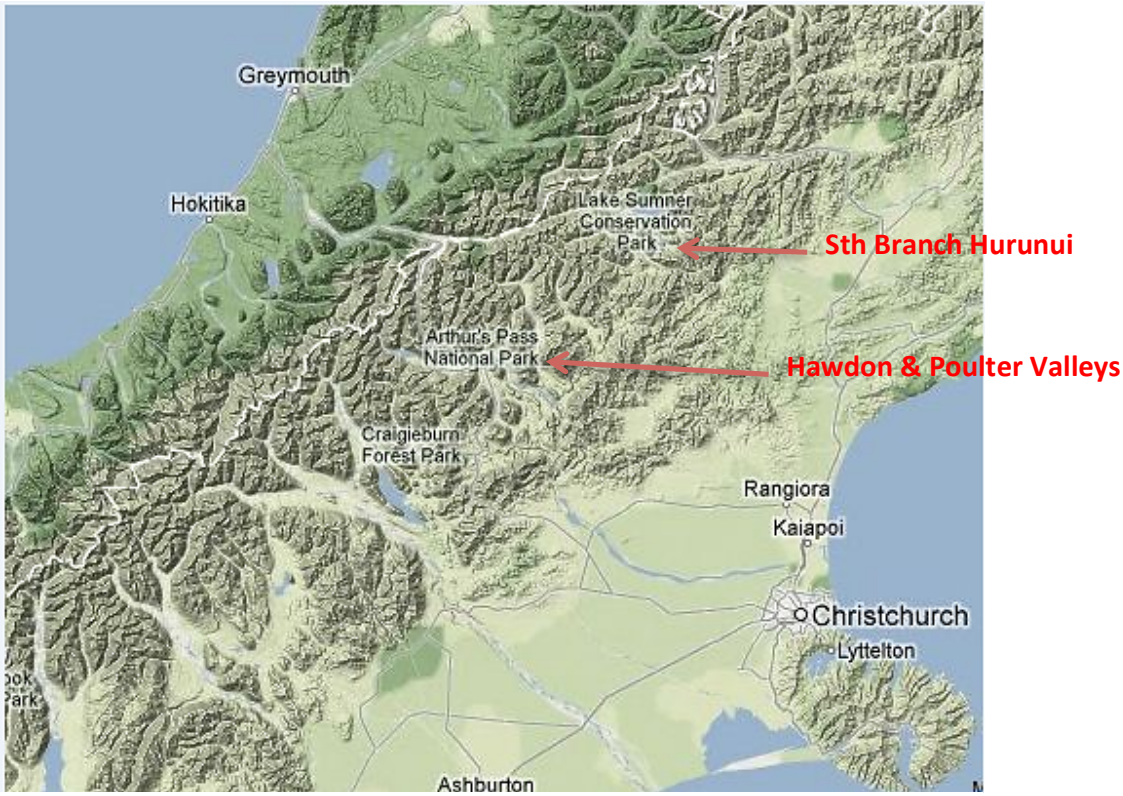

Fig 2: Hawdon &Poulter Valleys (Arthur's Pass National Park) & Sth Branch of the Hurunui (Lake Sumner Conservation Park), North Canterbury

Factors contributing to declines in *C. malherbi* range and abundance

Forest dependent birds and especially those that nest within tree cavities are particularly vulnerable to forest clearance (Innes et al. 2010). Whilst large areas of native forest do exist within New Zealand's National Parks many forest birds have become extinct or are threatened in these areas as forest age determines the availability of cavity-nesting sites (Innes et al 2010; Rhodes et al. 2009). Species, such as *C. malherbi* which nest in mature and dead *Nothofagus* trees are therefore adversely affected by the harvesting of beech forests (Kearvell 2002).

A number of behavioural and life-history traits predispose many birds to predation from introduced mammalian predators such as tameness, naivety, flightlessness, low-fecundity, ground feeding, ground and cavity nesting (Innes et al. 2010; Phillips 2010). Predation by competent ground and arboreal rodents and mustelids on female cavity-nesting NZ birds such as the saddleback (*Philesturnus spp*), kaka (*Nestor spp*), kākāriki (*Cyanoramphus spp*), and mohua (*Mohoua ochrocephala*) result in an excess of males and as these birds are all relatively long-lived, predation is expected to have adverse effects on their population size (Innes et al. 2010). Risks increase in species with long incubation periods, those which nest in late summer when predators may be abundant and are ground-feeders (Innes et al. 2010). Red crowned parakeets (*C. novaezelandiae*) for example, spend significant amounts of time on the ground and cavity-nesting is thought to have, contributed to the disappearance of *C. novaezelandiae* from mainland forests (Greene 1998; Taylor 1985). Cavity-nesting and feeding from low-growing vegetation make *C. malherbi* vulnerable to both native avian and exotic mammalian predators (Rhodes et al. 2009; Duncan & van Hal 2004; Kearvell 2002). In addition, female *C. malherbi* incubate up to ten eggs for 21-26 days from late summer with chicks remaining in the nest for 40-50 days (Duncan & van Hal 2004).

During beech mast years the breeding season may extend over autumn and winter (Duncan & van Hal 2004). Declines in populations of many hole-nesting forest birds follow periodic irruption of rodent (*Rattus spp.*) and stoat (*Mustela erminea*) populations in beech forests. Beech trees flower and seed heavily every 3-5 years and the resulting increase in food supply causes irruptions of introduced mice (*Mus musculus*), rats and stoats. Stoats are particular prolific breeders, with females producing up to 13 offspring a season when rodent numbers are high (Pryde et al. 2005). Consecutive beech masts over the 1999-2001 summers resulted in a rat irruption with the subsequent reduction in both the Hurunui population of *C. malherbi* (Duncan & van Hal 2004).

Sympatric endemic and introduced mammalian, avian and invertebrate species are known to compete for food and nesting resources in mainland forests (Innes et al. 2010; Towns 2012) As the availability of nesting sites plus the quality and quantity of food resources during the breeding season determines productivity, competition for resources is likely to contribute to the decline in native forest bird populations (Innes et al. 2010). *C. malherbi* numbers can increase substantially during mast years when up to three clutches can be produced (Kearvell pers. comm). However, beech seeds are also consumed by introduced birds and rodents and the abundant food supply increases the rate of predation by rats and stoats (Innes et al. 2010; Kearvell et al. 2002). The widespread and abundant brushtail possum (*Trichosurus vulpecula*) is also of conservation concern as these arboreal mammals consume large quantities of flowers, fruit and predate on nesting birds (Elliot et al 2010; Innes et al. 2010). Additionally, *Vespula* wasps are known to consume honeydew and invertebrates which are both important food sources for native forest birds in South Island *Nothofagus* forests during the spring (Innes et al. 2010; Duncan & van Hal 2004; Kearvell

2002) Extensive browsing of the forest understorey by ungulates, possums and hares has significantly altered the forest structure resulting in not only the loss of *C. malherbi*'s natural foraging sites but also refuges from predators (Duncan & van Hal 2004).

Avian diseases pose a potential threat to *C. malherbi* populations. The beak and feather disease virus (BFDV) which causes the potentially fatal psittacine beak and feather disease (PBFD) was isolated from *C. novaezelandiae* on Little Barrier Island and Eglington Valley *C. auriceps* (Massaro et al. 2012). In 2009, some *C. malherbi*'s on Maud Island were showing symptoms consistent with PBFD. Results indicate that antibodies for PBFD were detected in *C. malherbi*'s from both Maud Island and the captive-breeding unit but subsequent screening has been inconclusive (Kearvell pers. comm)

Conservation management to date has focused on maintaining the viability of *C. malherbi* populations by mainland habitat restoration, predator control and translocation of individuals for captive breeding and establishing populations on off shore islands (Grant & Kearvell, 2001). The Department Of Conservation's "Operation Ark" began in 2004 to protect and restore South Island mainland beech forests following the rat irruption of 1999 / 2000 through intensive rat, stoat and possum control (Bird International 2012, *C. malherbi*). As part of this initiative, *C.malherbi* nests in the Poulter, Hawdon and the South Branch of the Hurunui valleys are protected by tin tree-wraps plus traps around each nesting tree. Populations in these valleys are closely monitored to determine the effectiveness of these ongoing predator controls (Birdlife International 2012). To prevent the spread of PBFD all captive breeding stock held at Peacock Springs are screened annually and captive-reared birds are all tested prior to translocation (Massaro et al. 2012). Also, screening of wild

endemic parrots throughout New Zealand but the Department of Conservation commenced in 2012 (Kearvell pers. comm.).

Female *C malherbi* returning to nest, Hawdon Valley

Photo: Kate Beer, Department of Conservation

C. malherbi pair in the Poulter Valley

Photo: Department of Conservation

The objectives of this project were to:

1. To develop and characterize microsatellite loci for *C. malherbi* and cross-amplify these in *C. auriceps*.
2. To use microsatellite genotype data, and nuclear and mitochondrial sequence data to quantify genetic diversity within and genetic differentiation among the Hurunui, Poulter and Hawdon populations of *C. malherbi*
3. To determine the suitability of Poulter Valley *C. malherbi* individuals as an additional source population for captive breeding and subsequent translocation to offshore islands.
4. To use microsatellite genotype data, and nuclear and mitochondrial sequence data to determine the genetic distinctiveness of *C. malherbi* and *C. auriceps* and to identify cryptic hybrids within sympatric populations of these two species.
5. To provide useful genetic information to inform active conservation management of captive and translocated populations of the critically endangered *C. malherbi.*

Sampling and Methods

Non Invasive sampling

Noninvasive sampling from biological material such as eggs and shed feathers enables DNA to be obtained from free-ranging animals when extracting blood and tissue is not feasible as individuals are rare, difficult to locate and capture or are exceptionally sensitive to handling (Maurer et al. 2010; Beja-Pereira et al. 2009; Hogan et al. 2008). Successful extraction of sufficient DNA from shed feathers for population genetic studies has been achieved in a number of bird species. For example, Bayard de Volo et al. (2008) obtained relatively high yields of DNA from molted Northern Goshawk (*Accipiter gentuis*) feathers. Hogan et al. (2008) successfully extracted DNA from shed feathers of the cryptic and difficult to catch Australian nocturnal powerful owl (*Ninox strenua).* These authors concluded that successful

amplification of both mitochondrial and nuclear markers was influenced by feather quality rather than feather type. As the quality and quantity of extracted DNA influences the reliability of results these authors recommended discarding poor quality feathers. In this study, opportunistically collected feathers, eggs, embryos and dead birds were collected from the three wild populations and the captive population at Peacock Springs. Due to the limited number of samples available DNA was extracted from visibly degraded feathers and tissues.

Choice of Molecular tools

Microsatellites

Microsatellites are small tandem repeats of 1-6 nucleotides (e.g. CGACGACGACGA) found primarily in nuclear DNA and are widely used in population genetic studies (Selkoe & Toonan 2006; Balloux & Lugon -Moulin 2002). These selectively neutral, codominant, highly polymorphic markers, which follow a Mendalian mode of inheritance are found throughout the genome and are particularly useful for studying genetic variation and population structure (Selkoe & Toonan 2006). The conserved DNA sequences flanking the microsatellite are used to design species-specific primers for the amplification of each individual locus with polymerase chain reaction (PCR). In addition, as flanking regions may be conserved in closely related species, amplification of additional microsatellite loci is possible from previously designed primers (Selkoe & Toonan 2006). Microsatellites were particularly useful for this study as these markers can be amplified from small amounts of tissue including degraded samples and biological material obtained from non-invasive sampling (Selkoe & Toonan 2006). Furthermore, it was possible to include four additional microsatellite loci previously developed for Forbes' parakeet (*C. forbesi*) and successfully amplified in *C. malherbi* by Chan et al. (2005). This multilocus study incorporating

polymorphic loci will increase the power to estimate genetic diversity and population structure in *C. malherbi* (Selkoe & Toonan 2006). Cross-amplification of both *C. malherbi* and *C. forbesi* microsatellite loci in *C. auriceps* individuals was carried out to genetically differentiate between, and identify any cryptic hybrids within sympatric populations of *C. malherbi* and *C. auriceps.* Microsatellite data combined with Bayesian assignment tests remain a common method for hybridisation studies as the allele frequency differences between species enables individuals to be assigned to a species and the identification of hybrids (Väli et al. 2010)

The isolation of microsatellite markers from sequence data for taxa with few existing genetic resources has become faster and more cost effective with the development of next-generation sequencing technologies (Ekblom & Galindo 2011; Guichoux et al. 2011; Perry & Rowe 2011). In this study, I used Roche 454 pyrosequencing of extracted genomic DNA and the software program MSATCOMMANDER (version 0.8.2, Faircloth 2008) to search sequence data for microsatellite repeats and suitable flanking regions for primer design in *C.malherbi.*

Single nucleotide polymorphisms (SNPs)

Single nucleotide polymorphisms (SNPs) occur when a single base in a DNA sequence differs between individuals. These mostly bi-allelic, co-dominant markers are the most abundant and widely distributed source of sequence variation within genomes with one occurring about every 500 bp in animal populations (Garvin et al. 2010; Morin et al. 2004; Brumfield et al. 2003) and are therefore useful markers for population genetic studies (Belfiore et al. 2003). The biallelic nature of SNPs means that these markers have less power than microsatellites to estimate genetic diversity and fine-scale population structure, but this can be overcome by using more SNP loci (Morin et al. 2004; Blumfield et al. 2003). Despite being

less variable than microsatellites, SNPs are useful in conservation genetics as SNPs can be obtained from partially degraded DNA from poor quality historical, non-invasive or degraded samples plus only require the amplification of very short fragments (Helyar et al. 2011; Allendorf & Luikart 2007). Species-specific (diagnostic) SNPs are particularly useful for assigning individuals to a species, the identification of hybrids and the detection of introgression (Kalinowski 2010; Väli et al. 2010). As SNP genotypes are represented by A, T, C or G unlike microsatellites which are based on allele sizes, SNP data can be standardized and therefore direct comparison between SNP studies is possible (Morin et al. 2004).

Two nuclear exons (RAG-1 & CMOS) and mitochondrial (cytochrome *b*) genes were selected for this study as sequence data from closely related *Cyanoramphus* parakeets were readily available for all three genes in GENEBANK. The sequences were then aligned to locate putative species-specific (diagnostic) SNPs to genetically differentiate between *C. malherbi* and *C. auriceps* (Chapter 4).

Data Analysis

A more detailed account of data analyses and the programs used to perform these are included in the relevant chapters.

Genetic Diversity

In this study I quantified genetic diversity by calculating allelic diversity (the average number of alleles per locus), observed heterozygosity (H_O, the actual level of heterozygosity) and expected heterozygosity (H_E, the level of heterozygosity that would be expected under Hardy-Weinberg equilibrium). As multiple microsatellite loci were used in this study, I also tested for linkage-disequilibrium.-.the non-random association between alleles at different loci (Selkoe & Toonan 2006). Mutations in primer-binding regions may prevent amplification of alleles at a particular locus (null alleles) resulting in an apparent excess of homozygotes

relative to Hardy-Weinberg proportions (Selkoe & Toonan 2006). I therefore, used a statistical approach to estimate null allele frequencies in the microsatellite data set.

Population Structure

I assessed patterns of genetic differentiation in three complementary ways. Firstly, I used traditional estimates of genetic differentiation based on F_{ST} (Weir & Cockerham 1984) which was calculated via an analysis of variance (AMOVA). F_{ST} is based on allele frequency differences at each locus among the sampled populations and is useful for identifying population structure (Sunnuck 2011). However, F_{ST} requires that individuals are grouped prior to calculating differentiation and therefore, assumptions must be made about population structure (Sunnucks 2011). In addition F_{ST} assumes no selection, random mating, mutation-drift equilibrium, an island model of migration, an infinite allele model of mutation, and non-overlapping generations (Stow & Magnusson 2012). Data collected from natural populations within fragmented habitats are likely to violate most of these assumptions (Stow & Magnusson 2012). Slatkin (1995) developed an analogue of F_{ST} (R_{ST}) because microsatellites most commonly follow the stepwise mutation model. However, previous studies have shown that F_{ST} generally performs better than R_{ST} when samples sizes are small ($n \leq 10$), there are less than 20 loci and when populations are weakly structured (Balloux & Lugon-Moulin 2002; Gaggiotti et al 1999). For microsatellite data I also calculated F'_{ST} to correct for within-population diversity (Meirmans & Hedrik 2011). F'_{ST} provides a population-specific F_{ST} value rather than global or pairwise F_{ST} values and thus allows for differences in local population size and migration rate but is not affected by the mutation rate (Allendorf et al. 2012; Meirmans & Hedrik 2011). I have reported both F_{ST} and F'_{ST} values as F_{ST} values are valuable for comparative analysis, as a fixation index and are appropriate for biallelic markers such as SNPs. (Meirmans & Hedrik 2011). For sequence

data it is not necessary to calculate F'_{ST} as F_{ST} assumes two alleles at each locus (Meirmans & Hedrik 2011). For cytochrome *b*, ϕ_{ST} was calculated by using the distance-matrix of pairwise nucleotide-substitution differences between haplotypes. ϕ_{ST} was developed for sequence data where only the presence or absence of an allele is known (Meirmans & Hedrik 2011).

To determine the critical values for all multiple pairwise comparisons I used the Benjamini and Yekutieli (B-Y) method as outlined in Narum (2006).This modified false recovery rate (FDR) method increases the power to detect differentiation between pairs of populations and is particularly relevant to conservation genetics (Narum 2006).

Secondly, I used a Bayesian clustering method to infer populations or clusters of individuals based on each individual's multilocus genotypes (Sunnucks 2011; Manel et al. 2005). Groups are defined by the data and therefore this method is useful when population boundaries are not clearly defined (Manel et al. 2005). In this study I used the Bayesian clustering algorithm implemented in STRUCTURE (version 2.3; Pritchard et al. 2000) which assumes that populations are in Hardy-Weinberg equilibrium and that there is linkage equilibrium between loci, conditions that are not often met in natural populations (Rutledge et al. 2010; Hubisz et al. 2009) In STRUCTURE I used the admixture model which assumes that all individuals may have mixed ancestry which most likely reflects data from natural populations (Pritchard et al. 2010). I also used the correlated allele frequency model developed by Falush et al. (2003a) for closely related populations where it is expected that allele frequencies will be similar. In addition, I used sampling location as a prior (LOCPRIOR). This model uses sampling locations to assist in cluster assignment and was designed for the detection of structure when sample sizes are small (Hubisz et al. 2009).

Thirdly, I used a Principle Components Analysis (PCA) to determine population structure based on microsatellite genotype data. This non-Bayesian, multivariate ordination method makes no assumptions regarding Hardy-Weinberg equilibrium or linkage disequilibrium and does not require a prior definition of population structure (Rutledge et al. 2010). As natural populations do not have clear geographic boundaries a PCA allows for the data to define population structure and patterns of multilocus genetic variation to be visualised on a 2 dimensional scatterplot, with putative subpopulations forming distinct clusters (Reeves & Richards 2009).

Detection of hybridisation

I used a Bayesian assignment test to discriminate between species, detect hybridisation and identify hybrid individuals using multilocus microsatellite data. The clustering method implemented in STRUCTURE uses allele frequency differences between species and does not require data from a parental (allopatric) reference population (Väli et al 2010; Vähä & Primmer 2006).It is possible therefore, for hybrids between *C. malherbi* and *C. auriceps* to be detected as such assignment tests can rely on the analysis of mixed sympatric populations alone (Väli et al 2010).

Detection of recent migrants

In addition to the above analyses, assignment tests are also useful for determining the probability of a particular individual originating from the population from which it was sampled and therefore to identify recent migrants (Manel et al. 2005).

Structure of the Thesis

This thesis comprises five chapters: a general introduction, one paper, and two data chapters, a general discussion and an appendix. One paper has been accepted for publication in "Conservation Genetic Resources." Another paper is to be written in

collaboration with multiple authors during the early part of 2013. All the laboratory work and data analysis has been performed by me, with support from my supervisors and co-authors in the form of advice, technical support and discussion.

Chapter One: Introduction

The general introduction covers the background of conservation genetics, a short description of the genus *Cyanoramphus* and *C. malherbi* in particular and the main objectives of this project. I outline the factors which have contributed to the decline in numbers and range of *C. malherbi* and briefly describe past and on-going conservation management of this critically endangered parakeet. I have also outlined the non-invasive sample collection, molecular markers and data analysis used in this study.

Chapter Two: Characterisation of microsatellite loci in the critically endangered orange-fronted parakeet (Cyanoramphus malherbi) isolated using genomic next-generation sequencing.

In this chapter, I outline the sampling and methodology used to develop and characterise *C. malherbi* microsatellite loci for on-going and future population genetic studies. This paper has been accepted for publication in the journal "Conservation Genetic Resources."

Chapter 3: Genetic diversity and population structure of North Canterbury populations of the orange-fronted parakeet (Cyanoramphus malherbi)

This chapter describes the use of microsatellite genotype data, and nuclear and mitochondrial sequence data to estimate the population genetic structure among the Hurunui, Poulter and Hawdon populations to determine the suitability of Poulter Valley as an additional source population for captive breeding and subsequent translocation to offshore islands.

Chapter 4: The genetic distinctiveness of sympatric populations of the orange-fronted kākāriki (Cyanoramphus malherbi) and yellow-crowned parakeet (C. auriceps) in North Canterbury

In this chapter I describe the use both nuclear and mitochondrial DNA sequence and microsatellite genotype data to determine the genetic distinctiveness of *C. malherbi* and *C. auriceps* and to identify cryptic hybrids within sympatric populations of these two species.

Chapter Five: General discussion and conclusions

This section provides a critical evaluation of the results of the preceding chapters, a summary of the overall findings and suggestions for future research.

C. malherbi Blumine Island

Photo: John Kearvell, Department of Conservation

Chapter 2: Isolation and characterisation of microsatellite loci in the critically endangered orange-fronted kākāriki (*Cyanoramphus malherbi*)

Abstract

Cyanoramphus malherbi is a critically endangered endemic parakeet, or kākāriki, restricted to three mainland valleys in North Canterbury and four translocated populations on predator-free offshore islands of New Zealand. Using 454 sequencing of genomic DNA to identify microsatellites, a total of 16,497 sequenced fragments were obtained. Primers were designed for 35 of the 62 detected loci. Of these, 26 were amplified successfully and 18 were found to be polymorphic. Sequence homology was utilised to assign chromosomal locations in the assembled chicken, zebra finch and turkey genomes. Five loci were assigned to five different autosomes. No loci were found to be sex-linked. The number of alleles per locus ranged from two to seven with observed heterozygosities ranging between 0.09 and 0.70. All 18 loci readily cross-amplified and were polymorphic in *C. auriceps*, indicating the utility of these markers for conservation genetic management of both species.

The New Zealand critically endangered endemic orange-fronted parakeet or kākāriki (*Cyanoramphus malherbi*) was once widely distributed throughout the South Island as well as Stewart Island, but has never been observed in large numbers and consequently is considered to be the rarest of New Zealand's *Cyanoramphus* kākāriki (Taylor et al. 1986; Birdlife International 2012a). As a result of habitat loss, predation by introduced mammals, and competition for resources from both native and introduced species, small breeding populations of *C. malherbi* are currently restricted to three valleys in North Canterbury (Kearvell et al. 2002). A captive breeding programme has resulted in the establishment of four translocated populations on predator-free off-shore islands (Birdlife International

2012a). Microsatellites were developed to investigate the genetic diversity and population genetic structure of *C.malherbi* populations and to test the cross-utility of these markers in its near threatened congener, the yellow-crowned kākāriki (*C. auriceps;* Birdlife International 2012b).

Genomic DNA was extracted from feather and tissue collected from individuals of known provenance in both wild and captive populations using a DNeasy Blood and Tissue Kit (QIAGEN). Extracted DNA from a single bird was sent to the University of Otago for Genomic shotgun sequencing using the Genome Sequencer FLX (GS-FLX) system (Roche) following the protocol for a $1/16^{th}$ run as outlined in Abdelkirim et al. (2009). A total of 16,497 sequenced fragments ranging between 70 and 270 bp in length were obtained and converted into a single FASTA file and screened for di-, tri- and tetranucleotides with at least 6 repeats using MSATCOMMANDER (version 0.8.2; Faircloth 2008). In the 16,497 reads, 62 microsatellites were detected: 6 tetranucleotides, 10 trinucleotides and 46 dinucleotides. Primer pairs were developed using PRIMER3 (version 0.4.0; Rozen and Skaletsky 2000) for 35 microsatellite markers with sufficient flanking region for primer design.

To test for amplification and polymorphism, PCR amplifications were performed for five *C.malherbi* in 15 µl reactions containing: approximately 10 ng of genomic DNA, 1x NH4 reaction buffer (Bioline), 2mM $MgCl_2$, 0.2mM dNTPs, 0.4µM of each primer, and 1U BIOTAQ DNA polymerase (Bioline) using the following temperature profile: initial denaturation at 94°C for 4 min; 10 cycles at 94°C for 15s, Ta (°C) for 30s (see Table 1), 72°C for 30s, followed by 25 cycles at 89°C for 15s, Ta (°C) for 30s, 72°C for 30s, then a final extension of 72°C for 10 min. All reactions were run in a Mastercycler EP thermocycler (Eppendorf), and PCR

products were visualised by electrophoresis on a 2% agarose gel prestained with SYBRsafe (Invitrogen).

Twenty six loci were fluorescently labelled (forward primers labelled with 6-FAM, VIC or PET; ABI) and screened for polymorphism. Genotyping was performed on an ABI Prism 3130xl Genetic Analyser (Applied Biosystems). Allele sizes were determined using the size standard Genescan® 500-LIZ (Applied Biosystems), and scored visually using GeneMarker (version 1.9; SoftGenetics). Of the 26 loci, 18 were polymorphic in both *C. malherbi* and *C. auriceps* (Table 1), four monomorphic and four were too difficult to interpret.

To identify possible sex-linked microsatellite loci, molecular sexing of all birds was performed to amplify sex-specific introns (*CHD1W* and *CHD1Z*) using the protocol developed by Fridolfsson & Ellegren (1999) and subsequently optimised by Tokunaga et al. (2007) for sexing of the Forbes' kākāriki (*C. forbesi*). Captive birds of known sex were used as positive controls. PCR amplifications were carried out on a Mastercycler EP thermocycler (Eppendorf) in 15 µl reactions containing: 1x NH4 reaction buffer (Bioline), 2mM $MgCl_2$, 200µM dNTPs, 2pmol of primers 2550F and 2718R (Fridolfsson & Ellegren 1999), and 0.25 U BIOTAQ DNA Polymerase (Bioline). Thermal cycling was performed as follows: initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30s, primer annealing at 47°C for 30s, and product extension at 72°C for 1 min, before a final prolonged extension step of 72°C for 5 min. The PCR products were visualised on a 3% agarose gel stained with SYBRsafe (Invitrogen), with males displaying a single band from the *CHDZ1* intron and females displaying two bands from the *CHDW1* and the *CHDZ1* introns, respectively. Preferential amplification of the *CHDZ1* product over the *CHDW1* product may result in the inaccurate scoring of females as males (Tokunaga et al. 2007). Repeat PCRs

were therefore carried out when only the *CHDZ1* product amplified. As no evidence of sex linkage was detected, all 18 polymorphic microsatellite loci were included in the analyses below.

Chromosome locations of all 18 loci were tested by comparing *C. malherbi* microsatellite sequences with their homologs on the chicken (*Gallus gallus*; Hillier et al. 2004), zebra finch (*Taeniopygia guttata*; Warren et al. 2010) and turkey (*Meleagris galloparo*; Dalloul et al. 2010) genome assemblies in NCBI BLAST using the default parameters for the discontiguous megablast search algorithm. Five loci were assigned a chromosomal location with a BLAST hit E-value of > 1E-05 and over 70 base pair in length (Dawson et al. 2012; Table 1).

All 18 microsatellite primer pairs tested were polymorphic for both species with the number of alleles per locus ranging from two to seven in *C.malherbi* and two to five in *C.auriceps* (Table 1). Estimates of genetic diversity, tests for significant deviations from Hardy-Weinberg equilibrium and significant linkage disequilibrium were calculated using ARLEQUIN version 3.0 (Excoffier et al. 2005). Statistical tests were corrected for multiple comparisons using the Benjamini and Yekutieli (B-Y) correction (Narum 2006). Three loci (OFK9, OFK34 and OFK41) in *C. malherbi* significantly deviated from Hardy-Weinberg equilibrium showing a homozygote excess (B-Y corrected $P < 0.014$). Subsequent analyses of all 18 loci for both species using MICROCHECKER version 2.2.3 (van Oosterhout et al. 2004) indicated null alleles may be present at OFK34 in *C. malherbi* due to a general excess of homozygotes for most allele size classes. Significant linkage disequilibrium was detected in four pairs of loci (B-Y corrected $P < 0.008$; OFK9 and OFK33, OFK33 and OFK55, OFK33 and OFK 61, OFK19 and OFK 60) in *C.malherbi* but none were detected in *C. auriceps*. In addition, OFK 19 and OFK 60 were found to be located on chromosome 4 and 7, respectively (Table1).

Given the high synteny observed in birds (e.g., Warren et al. 2010), these combined data suggest that physical linkage for any of these loci is unlikely.

The microsatellite loci characterised in this study are proving to be a valuable resource for on-going population genetic studies of threatened kākāriki. The assessment of genetic diversity and population genetic structure of *C. malherbi* and detection of limited hybridisation between *C. malherbi* and *C. auriceps* is are informing active management strategies of captive and translocated populations of critically endangered *C. malherbi.*

Table 1: Characterisation of microsatellite loci developed for *Cyanoramphus malherbi*

						C.malherbi					
Locus	Repeat Motif	Primer Sequence (5'-3')	Forward primer label	Ta (°C)	Allele Size range (bp)	*n*	No. of alleles	H_O	H_E	HWE P-value	Chromosome, Location (E-value)
OFK9	$(AT)_8$	F: ATT CTC TGC TCC TGG GAT TG R: AGCCTGTGGGAGTGAGTGAT	PET	59	160-167	23	5	0.54	0.69	**0.002**	
OFK12	$(AC)_9$	F: AAAAGGCAAGCACTGAGGAG R: CCAAGGCTGGAATAACCTGA	6-FAM	60	192-220	22	2	0.09	0.16	0.131	Tgu1A (1e-52)
OFK19	$(CT)_7$	F: ACCAACCCCACCATTACCTT R: GACAGAAACCTGCTGTGTGC	VIC	60	117-124	23	2	0.25	0.22	1.000	Tgu4 (2e-31)
OFK21	$(AT)_8$	F: GGA AAA TCC AGT GTG GCT TC R: CTT TCT CTG CCA TGG AGC AT	VIC	60	158-164	21	3	0.48	0.42	0.394	
OFK26	$(GT)_{12}$	F: AGG CCT TTA TTG CTT GGT TG R: GGT TTG TAT CGG GCA GGT AG	VIC	60	116-126	22	3	0.14	0.28	0.025	
OFK31	$(CT)_7$	F: CAAGGAGGCGACTGGATTAC R: GCAGAATTCCTCCCAAACCT	6-FAM	60	98-106	22	3	0.30	0.27	1.000	Tgu24 (1e-84) Gga24 (7e-55)
OFK33	$(AC)_8$	F: TTGGGGTTTTTGTAAAGCATCT R: CGCCATAGGACAATAAACCA	6-FAM	59	222-236	21	3	0.18	0.17	1.000	
OFK34	$(AT)_9$	F: GAAAATGAATTCCTTAATTCCTTAAA R: TGCAAGATCTGGATTCTTTATACAT	PET	58	104-112	21	5	0.33	0.67	**0.0006**	
OFK41	$(ATTT)_7$	F: GCATTGTGGTGGTGGTGTTA R: TCAGTGAAGTGCTGGCTCTG	6-FAM	60	168-178	23	5	0.70	0.71	**0.001**	
OFK50	$(TA)_9(AT)_2$	F: CTTCTGACAATAGCTTATATAGACACA R: CCAGGAATTGCAGTAAACCAA	6-FAM	60	111-120	23	4	0.41	0.40	0.069	
OFK52	$(CCT)_6$	F: TTCAGTGGAAAAACAAGTACCC R: AAGCTGAGGAAAGCGATGAA	6-FAM	59	75-80	23	2	0.08	0.15	0.127	Tgu2 (4e-52) Gga2 (4e-39) Mga2 (2e-35)
OFK54	$(ATTT)_6$	F: TTCATCTCTCGGCTGCATAA R: CCCCATTTACTTCTCCATTTCA	PET	60	131-139	23	3	0.58	0.68	0.236	
OFK55	$(AGC)_6$	F: TGCAATCAGCGAGGTATTTTT R: GCTAAGCTACGTGGTGAAGTCC	VIC	57	111-120	21	4	0.40	0.39	0.738	
OFK56	$(TTTG)_5$	F: CAGTGTAGCTGGGGGTAGGA R: AGTTTTCCAACGCTTTGTGG	PET	60	172-185	21	3	0.14	0.14	1.000	
OFK58	$(GCA)_6$	F: CCACTCAACAAGGTGGGCTA R: CGGGCAGAAAGGGTACATAA	6-FAM	60	188-200	22	5	0.13	0.17	0.128	
OFK60	$(CA)_6(TA)_6$	F: GCTGATTAACCAGGGGAAAA R: AAAAAGTAAAAACAACAACCACCA	6-FAM	59	82-86	23	3	0.58	0.52	0.610	Tgu7 (4e-44) Gga7 (2e-40) Mga7 (2e-41)
OFK61	$(TC)_3(TC)_2(TC)_6$	F: AACGCACCCATCTCCTTCTA R: GAGGACTTAGTTGCGCCAAG	6-FAM	59	99-105	22	2	0.30	0.26	1.000	
OFK62	$(GT)_6(GT)_3$	F: AATTTTACAGGTACACATGTTGAGG R: GAGAGGTCCCCAATTCCTTC	6-FAM	60	107-110	22	2	0.08	0.08	1.000	

Chapter 3: Genetic diversity and population structure of North Canterbury populations of the orange-fronted kākāriki (Cyanoramphus malherbi).

Abstract

The critically endangered orange-fronted kākāriki (*Cyanoramphus malherbi*) is an endemic parakeet restricted to three small breeding populations within the Hawdon, Hurunui and Poulter valleys of North Canterbury, New Zealand. Since 2003, birds sourced from the Hawdon and Hurunui populations have been used for captive breeding and subsequent translocation to predator-free offshore islands. Discovered in 2003, birds from the Poulter valley had not been included in the captive breeding and translocation programme at the commencement of this project. To determine appropriate source populations for future translocation, this study used both nuclear and mitochondrial data to quantify the level of genetic diversity within, and the pattern of genetic differentiation among, the three remaining wild populations of *C. malherbi*. Six *C. malherbi* of known provenance from each of the three populations were genotyped at 18 microsatellite loci and sequenced at one mitochondrial (cytochrome *b*) and one nuclear (RAG-1) locus. For each valley, the number of microsatellite alleles ranged from one to four per locus. Observed and expected heterozygosities ranged from 0.0 to 1.0 and from 0.17 to 0.74, respectively. The Poulter valley had the highest average allelic diversity (2.3) and the highest average observed and expected heterozygosities (0.40 & 0.38, respectively). Weak but significant population genetic structure was detected among valleys (F_{ST} = 0.06, F'_{ST} = 0.09, p = 0.04). Pairwise F_{ST} estimates identified the Hurunui as being significantly different from both the Poulter and Hawdon Valleys (F_{ST} = 0.11, p = 0.01 and 0.061, p < 0.01, respectively). In contrast, STRUCTURE analysis indicated that the three valleys comprise a single genetic population.

Three cytochrome *b* haplotypes were identified, two of which were found in all three populations and one haplotype that was present in one Poulter valley individual only. Two RAG-1 alleles were identified, both of which were shared by all three valleys. No significant population genetic structure was detected for either the cytochrome *b* or RAG-1 loci. These combined data suggest that the three North Canterbury valleys function as a metapopulation with some level of connectivity. Incorporating individuals sourced from all three valleys into the captive breeding and translocation programme will help maximise the genetic diversity, and maintain the evolutionary potential of this critically endangered New Zealand endemic kākāriki.

Introduction

The translocation of endangered species to predator-free habitats outside their contemporary or historical range in order to establish new populations has become an important global conservation management strategy (Weeks et al. 2011). In addition to protecting species from the immediate effects of predation and on-going habitat loss, translocations may also mitigate the long-term detrimental genetic effects which may occur in small, fragmented and isolated populations (Weeks et al. 2011). However, the long-term viability of newly established populations depends on a number of environmental, demographic and genetic factors (Bouzat et al. 2009). The translocation of threatened New Zealand endemic species to protected mainland sites and offshore islands plays an important role in the conservation of declining mainland populations (Jamieson 2011; Towns et al. 2012). However, the size and quality of offshore islands habitats are likely to be sub-optimal relative to a species' mainland habitat (Cardoso et al. 2009). In addition, natural gene flow between newly established and mainland populations is unlikely as islands are

typically geographically isolated from source populations (Jamieson 2011). Consequently, if a small number of randomly selected and possibly related founders representing only a small fraction of the source population's genetic diversity are translocated to an isolated habitat with a small carrying capacity there is a risk of loss of genetic diversity, increased inbreeding and loss of evolutionary potential (Jamieson 2011). Furthermore, both natural and translocated island populations have been found to have lower levels of genetic diversity than mainland populations making them vulnerable to extinction (Boessenkool et al. 2007; Cardoso et al. 2009; Miller et al. 2011). Therefore, both the source and number of translocated individuals must be carefully considered when establishing and maintaining viable populations (Weeks et al. 2011). For many endangered species, captive breeding programmes have been established to augment overall population size plus to provide additional individuals for reintroductions and translocations when *in-situ* conservation is not feasible (Allendorf & Luikart 2007; Weeks et al. 2011; Towns et al. 2012). Many captive breeding populations have been established from a small number of founders of unknown relatedness which are unlikely to represent the natural genetic variation found in the source population (Frankham et al. 2010). In addition, only a small proportion of captive individuals successfully reproduce, further reducing genetic diversity (Frankham et al. 2010).Thus, to maximize genetic diversity, captive breeding programmes should ideally consist of unrelated founders from several populations with equal reproductive success (Alcaide et al. 2010; Jamieson 2011).

The New Zealand critically endangered endemic orange-fronted parakeet or kākāriki (*Cyanoramphus malherbi*) was once widely distributed throughout New Zealand, but has never been observed in large numbers and consequently is considered to be the rarest of New Zealand's *Cyanoramphus* kākāriki (Taylor et al. 1986; Birdlife International 2012). The

historical abundance and distribution of *C. malherbi* remains uncertain as a result of insufficient and possibly inaccurate historical identification (Triggs & Daugherty 1996; Nixon 1981; Harrison 1970) and the difficulty in accurately distinguishing between *Cyanoramphus* species from fossil remains (Holdaway et al. 2010). As a result of habitat loss and degradation, predation by introduced mammals, and competition for resources from both native and introduced species, small breeding populations of *C. malherbi* are currently restricted to native beech *(Nothofagus* spp) forests of the Hurunui, Hawdon and Poulter valleys of North Canterbury (Birdlife International 2012; Kearvell et al. 2002; Fig 1). During the 1999 and 2000 breeding seasons, the Hurunui population experienced substantial population declines in response to a rat irruption following two consecutive beech mast years (Bird Life International 2012). It is estimated that the total number of *C.malherbi* fell from approximately 500 - 700 birds to approximately 200 birds (Birdlife International 2012). Captive breeding of *C.malherbi* began in 2003 at Isaacs Wildlife Centre, Peacock Springs (Christchurch). Since 2005, captive-reared offspring from birds sourced from the Hurunui and Hawdon valleys have been translocated to predator-free Maud and Blumine Islands in the Marlborough Sounds, Chalky Island in Fiordland and Tūhua Island in the Bay of Plenty (Birdlife International 2012). The Poulter Valley population was discovered in 2003 and eggs from this valley were collected for captive breeding in 2009 (*C. malherbi* studbook) but as the genetic distinctiveness of this population had not yet been determined at the commencement of this project offspring of birds from the Poulter valley have not yet been selected for translocation. The objective of this project was to use microsatellite genotype data, and nuclear and mitochondrial sequence data to estimate the level of population genetic structure among the Hurunui, Poulter and Hawdon populations to determine the suitability of Poulter Valley as an additional source population for captive breeding and

subsequent translocation to offshore islands. This data combined with estimates of genetic diversity within the three remaining wild populations will help guide on-going conservation of *C. malherbi.*

Materials and Methods

Sampling

Feathers, dead adults, unhatched embryos and dead chicks were collected opportunistically from wild populations in the Hawdon, Hurunui and Poulter valleys by members of the Orange-fronted Kākāriki Recovery Group between 2004 and 2012. Feathers and dead birds were also obtained from the captive population at Peacock Springs. Blood and tissue samples previously collected from wild birds in the Hurunui and Hawdon valleys in 1996 were also obtained from Victoria University of Wellington. Given the importance of including unrelated birds of known provenance only and because the majority of samples in hand were related birds, the sample size for this study was restricted to 18 birds (see Appendix 1 for details). For example, despite having 57 samples in hand from the Hawdon and Hurunui valleys, 32 of these birds were offspring of one breeding pair and only 6 from each valley were unrelated. Based on the *C. malherbi* studbook, birds sourced from Peacock Springs were considered unrelated if they did not share grandparents. For wild samples, birds were considered unrelated if they originated from different nests. All samples are now stored permanently at the School of Biological Sciences, University of Canterbury.

DNA extraction

Genomic DNA was extracted using a DNeasy Blood and Tissue Kit (QIAGEN) according to the manufacturer's instructions. However, feather tips were incubated in buffer ATL (step 1)

overnight at room temperature. DNA quantity and quality were assessed with a NanoDrop ND-1000 spectrometer.

Microsatellites

All 18 individuals from the Hawdon, Hurunui and Poulter valleys were genotyped at 17 polymorphic microsatellite loci developed for *C. malherbi* (see Chapter 2) plus an additional microsatellite loci (Cfor 0809) developed for Forbes' parakeet (*C. forbesi*) and successfully amplified in *C. malherbi* by Chan et al. (2005) were screened on all 18 samples using the same PCR protocol developed for these loci. All reactions were run in a Mastercycler EP thermocycler (Eppendorf), and PCR products were visualised by electrophoresis on a 2% agarose gel prestained with SYBRsafe (Invitrogen). Allele sizes were determined using the size standard Genescan® 500-LIZ (Applied Biosystems), and scored visually using GENEMARKER (version 1.9; SoftGenetics).

Estimates of genetic diversity, tests for significant deviations from Hardy-Weinberg equilibrium, tests for significant linkage disequilibrium and estimates of population structure (F_{ST}) were calculated using ARLEQUIN (version 3.5; Excoffier & Lischer 2010). F'_{ST} was also calculated to correct for within-population diversity (Meirmans & Hedrik 2011). Multiple pairwise comparisons were corrected using the Benjamini and Yekutieli (B-Y) correction (Narum 2006). To infer the number of clusters (K) in *C. malherbi*, I used the Bayesian clustering algorithm implemented in STRUCTURE (version 2.3; Pritchard et al. 2010). Ten independent replicates (20,000 burn-in and 200,000 steps) with different random seeds were run for K = 1-4 using the admixture ancestry model, correlated allele frequency model and sampling location as a prior as per the recommendations by Hubisz et al. (2009) for small sample sizes and weak genetic structure. I determined the number of

clusters by comparing corrected estimates of the posterior probability, denoted *L*(*K*), for each value of *K*. To determine the probability of an individual originating from the population from which it was sampled (i.e., to detect recent migrants), I performed an assignment test in STRUCTURE using the parameters above. A Principle Components Analysis (PCA) to further visualise the pattern of genetic differentiation were performed using GENALEX (version 6.4.1; Peakall & Smouse 2006).

Mitochondrial cytochrome b

Mitochondrial cytochrome *b* gene sequences from *C. malherbi*, *C. auriceps* and *C. novaezelandiae* were previously studied by Boon et al. (2000) in an effort to resolve the taxonomy of New Zealand's *Cyanoramphus* parakeets. I aligned the three *C. malherbi* and six *C. auriceps* cytochrome *b* sequences available in GENBANK (Accession nos: AF218735-38, AF 218756-60; Boon et al. 2000) using GENEIOUS (version 5.5.4) to locate putative diagnostic sites as part of a study to differentiate between *C. malherbi* and *C. auriceps* (Chapter 4). These data could also be useful to investigate genetic diversity and population structure in *C. malherbi* so I also included them in this study. One putative diagnostic site was detected at position 729 with an adenine (A) in *C. malherbi* and a guanine (G) in *C. auriceps.* To target this site, forward and reverse primers (OFP cyt*b* F & OFP cyt*b* R, Table 1) were designed using PRIMER3 (version 0.4.0; Rozen and Skaletsky 2000) using the complete cytochrome *b* sequence of one *C. malherbi* individual (Accession no: AF218757; Table 1). PCR amplifications were performed in 25µl reactions containing: approximately 10ng of genomic DNA, 1x NH4 reaction buffer (Bioline), 2mM $MgCl_2$, 0.2mM dNTPs, 0.4µM of each primer, and 1U BIOTAQ DNA polymerase (Bioline) using the following PCR protocol: 95°C for 3 min, followed by 35 cycles of 95°C for 40s, 54°C for 30 s and 72°C for 60s. Sequencing was carried out using the BigDye® Terminator v 3.1 Cycle Sequencing kit on a 3130xl Genetic

Analyzer (Biosystems). As the sequence obtained using the reverse primer (OFP-cytbR) was difficult to read an internal reverse primer (OFK cytbR-internal; Table 1) was designed using PRIMER3. Cytochrome *b* haplotypes were determined by only aligning forward sequences in GENEIOUS (version 5.5.4; Biomatters). Estimates of population genetic structure (ϕ_{ST}) were calculated using ARLEQUIN (version 3.5; Excoffier & Lischer 2010).

Nuclear RAG-1

The single-copy nuclear recombination activating gene (RAG-1) *C. auriceps* and *C. novaezelandiae* (Accession nos: GQ505212 & 213) sequences previously published by Schweizer et al. (2010) to determine the evolutionary diversification of parrots were aligned to locate putative diagnostic sites as part of a study to differentiate between *C. malherbi* and *C. auriceps* (Chapter 4). These data could also be useful to investigate genetic diversity and population structure in *C. malherbi* so I included them in this study. A single polymorphic site was found at position 235. To amplify this site, a forward primer (Rag-1 F) was designed using PRIMER3 from the *C. novaezelandiae* sequence (Table 1). I used the reverse primer (R8, Table 1) designed by Groth & Barrowglough (1999) based on conserved chicken, human, mouse and Xenopus sequences. PCR amplifications were performed in 25µl reactions containing: approximately 10ng of genomic DNA, 1x NH4 reaction buffer (Bioline), 2mM $MgCl_2$, 0.2mM dNTPs, 0.4µM of each primer, and 1U BIOTAQ DNA polymerase (Bioline). The modified touchdown PCR protocol published in Groth & Barrowclough (1999) was as follows: an initial denaturation at 94°C for 10 min followed by five cycles of 95°C for 20 s, 61°C for 20 s, 72°C for 1 min. Two identical 5-cycle phases followed, with a 2°C reduction in annealing temperature for each phase. The final phase consisted of 25 additional identical cycles, with a final annealing temperature of 55°C. Sequencing was carried out in both directions using a 3130xl Genetic Analyzer (Biosystems). RAG-1

genotypes were determined by assembling the forward and reverse sequence for each sample and subsequently aligning consensus sequences in GENEIOUS (version 5.5.4; Biomatters). Estimates of population genetic structure (F_{ST}) were calculated using ARLEQUIN (version 3.5; Excoffier & Lischer 2010). The RAG-1 genotype data was also combined with the microsatellite data to determine population structure in ARLEQUIN and for both population and assignment tests in STRUCTURE (version 2.3; Pritchard et al. 2010).

Nuclear C-MOS

The single copy nuclear proto-oncogene (C-MOS) gene sequence from *C. auriceps* (accession no: GQ505104) previously used by Schweizer et al. (2010) to determine the evolutionary diversification of parrots, was aligned with the chicken (CHKMOS) sequence (Hillier et al. 2004) to locate putative diagnostic sites as part of a study to differentiate between *C. malherbi* and *C. auriceps* (Chapter 4). These data could also be useful to investigate genetic diversity and population structure in *C. malherbi* so I included them here. One polymorphic site was located at position 153. I used the forward primer (F944) designed by Cooper & Penny (1997) and designed the reverse primer (Cmos R) using PRIMER3 based on the *C.auriceps* sequence to amplify this target site (Table 1). PCR amplifications were performed in 25µl reactions containing: approximately 10ng of genomic DNA, 1x NH4 reaction buffer (Bioline), 2mM $MgCl_2$, 0.2mM dNTPs, 0.4µM of each primer, and 1U BIOTAQ DNA polymerase (Bioline.) The PCR protocol outlined in Schweizer et al. (2010) was modified by increasing the denaturation temperature by 4°C and the annealing temperature by 7°C. Sequencing was carried out in both directions using a 3130xl Genetic Analyzer (Biosystems). C-MOS genotypes were determined by assembling the forward and reverse sequence for each sample and subsequently aligning consensus sequences in GENEIOUS (version 5.5.4; Biomatters).

Table 1: Cytochrome *b*, C-MOS & RAG-1 primers used in SNPs' discovery

Primer name	Reference	Primer sequence (5' to 3')	Product size
OFP cytbF	This study	TCC TAA CTG GCC TGC TCC TA	542 bp
OFP cytbR	This study	GTT GGG TGA GAA GAG GGC TA	
OFP cytbR-internal	This study	GGG TAG TTA GTA GGA AGA GTA TAA	518 bp
Cmos F (F944)	Cooper & Penny, 1997	GCC TGG TGC TCC ATC GAC TGG	301 bp
Cmos R	This study	TTA CAT GGT GCA GGG TGA TG	
Rag-1 F	This study	GCA GAT CTT TCA GCC TTT GC	336 bp
Rag1 R (R8)	Groth & Barrowglough,1999	CTG ACA TCT CCC ATT CCG TCA CA	

Results

Microsatellites

All 17 *C.malherbi* and the *C. forbesi* microsatellite primer pairs tested were polymorphic with the number of alleles per locus ranging from two to four (Table 2). Four *C. malherbi* individuals (W016, SB280, FT3314 and FT3317) had 16% missing microsatellite data and five individuals (W026, FT3309, FT3315, SB138 and W013) had 0.05%, missing data. None of the 18 loci significantly deviated from Hardy-Weinberg equilibrium in any of the three populations (Table 2,) and therefore no loci were assessed for null alleles. Significant linkage disequilibrium was detected in four pairs of *C. malherbi* loci but these are unlikely to be linked (Chapter 2). Observed and expected heterozygosities ranged from 0.0 to 1.0 and from 0.17 to 0.74, respectively (Table 2) The Hawdon population had the lowest value of both observed and expected heterozygosity (0.29 & 0.33 respectively, Table 2) whilst the Hurunui

population had the lowest average allelic diversity (1.9,Table 2). Overall, the Poulter population had the highest average allelic diversity and the highest average observed and expected heterozygosities (2.3, 0.40 & 0.38, respectively, Table 2).

Table 2: Locus-specific summary statistics for all 18 microsatellite loci for each valley

	Hawdon					Hurunui					Poulter				
Locus	*n*	No. of alleles	H_O	H_E	HWE P-value	*n*	No. of alleles	H_O	H_E	HWE P-value	n	No. of alleles	H_O	H_E	HWE P-value
OFK9	6	2	0.67	0.54	1.00	6	4	0.33	0.56	0.19	6	3	1.00	0.62	0.09
OFK12	6	2	0.00	0.30	0.09	6	1				6	2	0.33	0.30	1.00
OFK19	6	2	0.17	0.17	1.00	6	2	0.17	0.17	1.00	6	2	0.50	0.41	1.00
OFK21	6	2	0.33	0.30	1.00	4	2	0.75	0.53	1.00	6	4	0.50	0.44	1.00
OFK26	6	2	0.00	0.30	0.09	6	1				6	3	0.33	0.53	0.52
OFK31	6	3	0.33	0.32	1.00	6	1				6	2	0.33	0.30	1.00
OFK33	6	1				6	1				5	3	0.60	0.51	1.00
OFK41	6	4	0.50	0.74	0.07	6	3	0.83	0.62	0.39	6	3	1.00	0.62	0.09
OFK50	6	2	0.50	0.41	1.00	6	2	0.33	0.30	1.00	6	2	0.33	0.30	1.00
OFK52	6	2	0.17	0.17	1.00	6	1				6	2	0.00	0.30	0.09
OFK54	6	3	0.50	0.71	0.20	6	3	1.00	0.71	0.58	6	2	0.50	0.53	1.00
OFK55	4	3	0.25	0.46	0.14	6	3	0.83	0.62	0.63	6	1			
OFK56	6	1				6	2	0.33	0.30	1.00	6	1			
OFK58	6	1				6	1				5	3	0.20	0.38	0.11
OFK60	6	3	0.67	0.67	0.72	6	2	0.33	0.30	1.00	6	2	0.67	0.48	1.00
OFK61	6	2	0.33	0.30	1.00	6	1				5	2	0.40	0.35	1.00
OFK62	6	1				5	2	0.20	0.20	1.00	6	2	0.16	0.16	1.00
Cfor0809	5	3	0.80	0.62	1.00	3	3	0.67	0.73	1.00	6	3	0.50	0.68	0.30
Average		**2.2**	**0.29**	**0.33**			**1.9**	**0.32**	**0.28**			**2.3**	**0.40**	**0.38**	

Abbreviations: number of individuals genotyped (n); observed heterozygosity (H_O); expected heterozygosity (H_E); test for significant deviation form Hardy-Weinberg Equilibrium (HWE).

An analysis of molecular variance (AMOVA) revealed that most of the genetic differentiation was within, not among, the three populations (93.7% and 6.3%, respectively; F_{ST} = 0.06, p = 0.04) and the corrected estimate of population-specific F_{ST} (F'_{ST}) = 0.09. Similar results were obtained when the RAG-1 data was included with microsatellite data (F_{ST} = 0.06, p < 0.02). Following B-Y correction, pairwise estimates of F_{ST} indicated weak but significant structure, with birds from the Hurunui being significantly different from both the Hawdon and Poulter birds (Table 3). STRUCTURE analysis provided no evidence of population genetic structure.

Table 3: Pairwise genetic differentiation (F_{ST} & F'_{ST}) among the Hawdon, Hurunui & Poulter populations based on Microsatellites

	Hawdon	Hurunui	Poulter
Hawdon			
Hurunui	**0.061 (0.054) F'_{ST} 0.09**		
Poulter	0.018 (0.021) F'_{ST} 0.03	**0.11 (0.10) F'_{ST} .0.16**	

Highlighted values are statistically significant (P < 0.027, B-Y correction). Figures in brackets: analysis includes both microsatellites and rag-1 data.

Of the four levels of K investigated, the highest average likelihood score was significantly higher for K = 1 (Fig 3). The principal component analysis (PCA) clustered individuals from the three valleys into three groups but with some overlap (Fig 4). Again, similar results were obtained when the RAG-1 data was included with microsatellite data (Appendix 2). The assignment test provided evidence of structure as all individuals were assigned to the population from which they were sampled (assignment probabilities ranged from 89-99%, Fig 5).

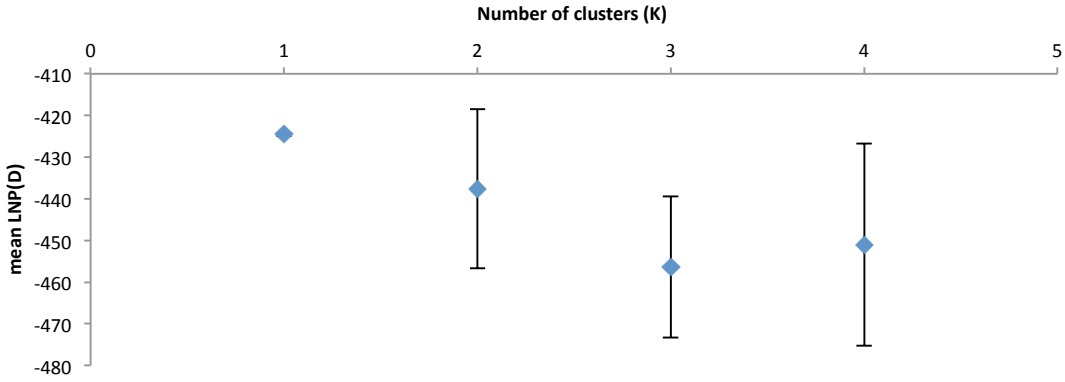

Fig 3: Determination of the most likely number of clusters (K) among Nth Canterbury *C. malherbi* samples based on microsatellite data. Plot of mean (± SD) log liklihoods of STRUCTURE analysis for K = 1-4

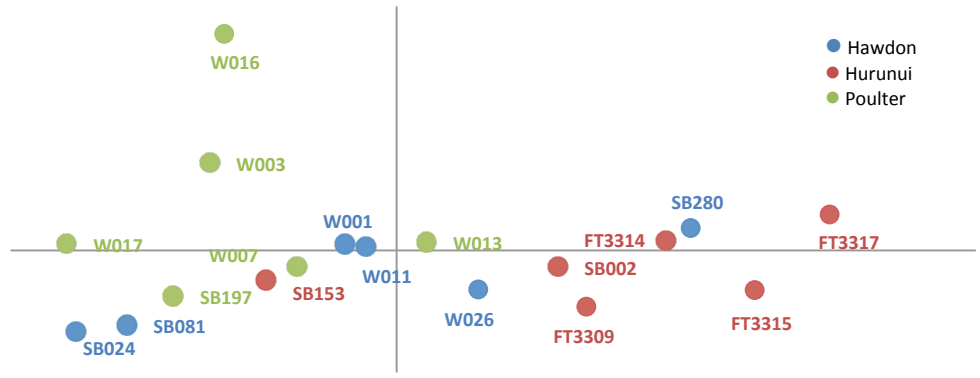

Fig 4: Principle Components Analysis (PCA) of multilocus genotypes based on microsatellite data of all *C. malherbi* individuals from each North Canterbury valley.

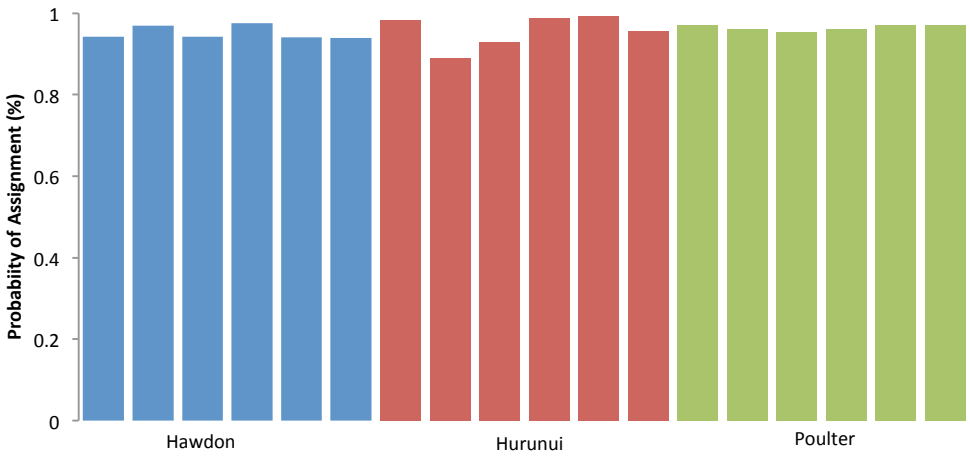

Fig 5: Assignment of individuals to their population of origin

Mitochondrial cytochrome b

All 18 samples from the Hawdon, Hurunui and Poulter valleys amplified and sequenced successfully. Despite using the internal reverse primer the majority of reverse sequences were difficult to read. Consequently, only the forward sequences were aligned. Four variable sites were found at positions 80, 154, 340 and 469 (all C↔T transitions) producing three haplotypes (A, B, C). Haplotypes A and C were found in all three valleys and haplotype B was found in one individual from the Poulter valley only (Table 4). No significant structure was detected by either global (Φ_{ST} = 0.054, p = 0.54) or pairwise comparisons among the three valleys (Table 5)

Table 4: Cytochrome b haplotype & RAG-1 genotype frequencies for each North Canterbury population.

Cyt*b* Haplotypes						Hawdon	Hurunui	Poulter
	80	154	340	469	538			
A	C	C	C	C	A	3	1	1
B	C	T	C	C	A	0	0	1
C	T	T	T	T	A	3	5	4

Table 5: Pairwise genetic differentiation (ϕ_{ST}) among the Hawdon, Hurunui & Poulter populations based on four cytochrome *b* SNPS

	Hawdon	Hurunui	Poulter
Hawdon			
Hurunui	-0.08		
Poulter	-1.31	0.067	

Nuclear RAG-1

Only one sample (W026 from the Hawdon) failed to amplify. The forward and reverse sequences of the remaining birds were assembled and aligned. Only one variable site was found at position 235 (C↔T transition). Sixteen birds were homozygous with either CC or TT and six were heterozygous at this site (Table 6). No individual from the Hawdon Valley had the TT haplotype (Table 3). No significant structure was detected by either global (F_{ST} = 0.04, p = 0.24) or pair-wise comparisons among the three valleys (Table 7).

Table 6: RAG-1 genotype frequencies for each North Canterbury population.

Rag 1 Genotypes	Hawdon	Hurunui	Poulter
235			
CC	4	4	2
CT	1	1	3
TT	0	1	1

Table 7: Pairwise genetic differentiation (F_{ST}) between the Hawdon, Hurunui & Poulter populations based on one Rag-1 SNP

	Hawdon	Hurunui	Poulter
Hawdon			
Hurunui	-0.021		
Poulter	-0.146	-0.025	

Nuclear C-MOS

Despite attempting to optimise amplification conditions the majority of samples failed to amplify reliably so C-MOS was excluded.

Discussion

The small sample size used in this study reflects the inherent difficulty of studying fragmented, remnant populations of critically endangered species (Struebig et al. 2012), particularly for species that are also cryptic and secretive (Landguth et al. 2012). Only 18 unrelated birds were obtained despite using samples collected prior to, and during, the three breeding seasons concurrent with my research. Whilst using such a small sample size may not detect all alleles present in a population, it is possible to achieve informative estimates of genetic diversity and allele frequencies by increasing the number of variable loci used (Hale et al. 2012; Landguth et al. 2012; Struebig et al. 2012).

Genetic Diversity

Whilst it is possible that my small sample size underestimates the actual genetic variation found in these three source populations (Hale et al. 2012), the estimates of allelic diversity (1 - 4 alleles per locus) and expected heterozygosity (0.17 to 0.74) in this study are consistent with other threatened bird species. Globally, threatened endemic birds that are known to have experienced demographic bottlenecks commonly have low allelic diversity (A) and low expected heterozygosity (H_E) (Jamieson 2009). For example, kakapo *Strigops habroptilus (A = 3.3, H_E = 0.47)*; takahe *Porphyrio mantelli* (A = 2.3, H_E = 0.33) (Jamieson 2009);; South Island robin, *Petroica australis australis* (A = 2.8, H_E = 0.36; Boessenkool et al. 2007); Mauritius kestrel *Falco punctatus* (A = 2.3, H_E = 0.42; Wenzel et al. 2012); and in South Pacific populations of peregrine falcons *Falco peregrinus* (A = 4.1, H_E = 0.49; Talbot et al. 2011). In contrast, based on eight microsatellite loci, native European populations of house sparrow (*Passer domesticus*) have a mean allelic diversity of 13.6 alleles and a mean expected heterozygosity of 0.86 (Schrey et al. 2011). In my study the three valleys had similar levels of genetic diversity with average allelic diversity ranging from 1.9-2.3 per locus

but overall, the Poulter Valley was found to have the highest level of genetic diversity (allelic diversity, heterozygosity plus all three cytochrome *b* haplotypes).

The cytochrome *b* diversity identified in this study is consistent with other critically endangered birds. For example, Steeves et al. (2010) found two haplotypes in the critically endangered black stilt (kaki*, Himantopus novaezelandiae*) and Rayner et al. (2010) identified five cytochrome *b* haplotypes in New Zealand's endangered Cook's petrel (*Pterodroma cookii*). In comparison, Chan et al. (2011) identified only one cytochrome *b* haplotype in the endangered Cook Island kakerori (*Pomarea dimidiata*), and the critically endangered Hawaiian Nihoa Millerbird (*Acrocephalus familiaris* k*ingi*) was also found to be monomorphic (Addison and Diamond 2011).

All three valleys shared the two RAG-1 alleles. Both the Poulter and Hurunui valley individuals had all three of the RAG-1 genotypes (CC, TT & CT) whereas the TT genotype was not found in the Hawdon Valley samples. Variation in the nuclear RAG-1 exon has extensively been used in avian phylogenetic studies (Griffiths et al. 2007; Treplin et al. 2008) but, to my knowledge has not previously been used for studies of genetic diversity and population structure. Consequently, whilst it is difficult to determine whether the RAG-1 variation identified in this study is consistent with other avian species, the genetic variation in this nuclear exon may have an important role in the long-term viability and evolutionary potential in *C. malherbi*

Previous research has demonstrated that mainland populations have greater genetic diversity than both natural and translocated island populations (Boessenkool et al. 2007; Jamieson et al. 2008; Cardoso et al. 2009; Miller et al. 2011). Therefore, to conserve the overall genetic diversity of *C. malherbi* on-going *in-situ* conservation of mainland source

populations is essential. In addition, as small numbers of individuals sourced from mainland populations for captive breeding and translocation are unlikely to represent overall diversity, it is imperative to consider both the source and number of individuals when establishing new populations (Frankham et al. 2010; Weeks et al. 2011). Whilst this study may have underestimated the overall genetic diversity of *C. malherbi* (but see above), the three cytochrome *b* haplotypes, two RAG-1 alleles and eighteen polymorphic microsatellite loci quantified provide a useful guideline for identifying both the source and number individuals to maximise the genetic diversity of captive and translocated populations.

Frankham et al. (2010) proposed that the genetic diversity of captive and translocated populations can be preserved by a founding population of 20 -30 breeding pairs. In contrast, Witzenberger & Hochkirch's (2011) literature review of the genetic management of *ex-situ* conservation programmes of a broad range of taxa suggested that a minimum of 15 founding individuals is sufficient to preserve 90% of natural genetic diversity over the next 100 years (an H_E of 0.54). While heterozygosity is important to conserve, further subdividing of populations during the establishment of captive and translocated populations will have a greater impact on allelic diversity than heterozygosity (Allendorf & Luikart 2007; Weiser et al. 2011). In order to establish long-term self-sustainable populations it is essential to conserve allelic diversity as this will determine the potential of fragmented populations to adapt to changes in habitat, climate or disease (Boessenkool et al. 2007; Caballero et al. 2010; Weiser et al. 2011). Tracy et al. (2011) suggest that conserving 95% of moderately rare alleles (frequency ≤ 0.05) in the threatened New Zealand mohua (*Mohua ochrocephala*) over 20 years would require the release of 60 individuals. These findings indicate that to conserve the overall genetic diversity of *C. malherbi*, would require a captive and

translocated population of approximately 30 breeding pairs. On the other hand, Taylor & Jamieson (2008) found changes in allele frequencies but little or no loss of genetic diversity in translocated populations of the South Island saddleback (*Philesturnus carunculatus carunculatus*). These authors concluded that threatened species such as the South Island saddleback with low levels of allelic diversity (average number of alleles per locus 2.7, H_E < 0.52) were at less risk of further losses of genetic diversity during founder events than genetically diverse species as common alleles are well represented in founding individuals. In addition, Taylor & Jamieson (2008) estimated that between 10 and 15 translocated breeding pairs were required to maintain the genetic variation in this threatened bird over the next 100 years, depending on initial allele frequencies.

Genetic variation in captive and translocated populations can also be maximized by having knowledge of the origin and genetic relationships of breeding pairs to minimize the likelihood of inbreeding and limiting the reproductive success of a few fecund individuals (Jamieson et al. 2008; Hagan et al. 2011; Witzenberger & Hochkirch 2011). Alcaide et al. (2010) suggested a significant loss of genetic variation occurred in reintroduced populations of the European lesser kestrel (*Falco naumanni*) due to large variances in the reproductive success of captive individuals. Jamieson (2009) proposed that as a result of small founder numbers, poor initial survival rates and the differential breeding success of individuals, levels of inbreeding increased in translocated populations of takahe *Porphyrio mantelli*, South Island robins (*Petroica australis australis*) and South Island saddleback (*Philesturnus carunculatus carunculatus*).

A minimum of 10 breeding pairs may maintain the genetic diversity of *C. malherbi*, but retaining evolutionary potential also requires the conservation of rare to moderately rare

alleles (frequency ≤ 0.05, Tracy et al. 2011). However, to conserve both rare and moderately rare alleles would require a large number of founders (Taylor et al. 2011) as mentioned above. As it may not be feasible to establish a population of 30 breeding pairs (Kearvell, personal communication), I recommend a captive and translocated population of at least 15 breeding pairs as suggested by Taylor and Jamieson (2008), consisting of unrelated birds of equal reproductive input from each of the mainland source populations in order to conserve microsatellite diversity, the three cytochrome b haplotypes and both RAG-1 alleles identified in this study.

Genetic Structure

The genetic clustering algorithm in the program STRUCTURE indicated that all of the sampled birds group into a single genetic population whilst AMOVA, PCA and the assignment test suggested weak genetic structure among the North Canterbury populations of *C. malherbi*. This discrepancy likely reflects the inability of the program STRUCURE to detect weak genetic structure (Hubisz et al. 2009; Miller et al. 2012). A recent study of the genetic structure of a Northern American population of the yellow rail (*Coturnicops noveboracencis*) also found a discrepancy between analyses in the presence of weak structure (Miller et al. 2012). The assignment tests provided no evidence of recent migration among the three populations but these findings may reflect the low power to resolve recent migration due to weak structure

The weak but significant genetic structure quantified in this study indicates that there is some degree of connectivity among the three North Canterbury valleys. Such interacting populations (probably acting as a metapopulation) are not uncommon in fragmented habitats (Allendorf & Luikart 2007; Armstrong and Seddon 2007). For example, weak but

significant genetic structure within a metapopulation of the threatened endemic NZ kaka, (*Nestor meridionalis*) suggests these forest-dwelling parrots disperse among nine remnant populations (Sainsbury et al. 2006). The significant genetic differentiation between the Hurunui and the Poulter and Hawdon populations may be explained by the distance (30km, Fig) between the Hurunui Valley within Lake Sumner Forest Park and the Hawdon and Poulter Valleys within Arthur's Pass National Park

The absence of stronger genetic differentiation among the three valleys may have a number of causes. Firstly, it is conceivable that the observed genetic structure among the three valleys has been underestimated. This would occur if gene flow was restricted among the valleys but insufficient time had elapsed for structure to be detected, my sample size was inadequate and/or the microsatellites were not variable enough. Since human settlement in New Zealand many endemic species have experienced substantial range contractions (Jamieson et al. 2006). The NZ endemic NZ forest-dwelling kaka (*Nestor meridionalis*) has had a similarly fragmented distribution as *C. malherbi* since this time (Sainsbury et al. 2006). A recent study of population structure among fragmented kaka populations also found weak genetic structure but these authors could not determine whether these results reflect insufficient time since fragmentation for genetic drift to have generated differentiation despite barriers to gene flow or whether fragmentation had limited dispersal among forest fragments (Sainsbury et al. 2006). It is possible therefore that these conclusions are also relevant to this study. As previously mentioned small sample sizes are often unavoidable in studies of fragmented populations and meaningful results can be achieved by increasing the number of microsatellites used (Struebig et al. 2012). It is unlikely that the microsatellites used in my study lacked power to detect genetic structure. Previous studies have

successfully detected structure using fewer than eighteen loci. For example, weak genetic structure was detected using 13 microsatellite loci in the white-ruffed Manakin (*Corapipo altera*, Barnett et al. 2008), eight loci in kaka (*Nestor meridionalis*, Sainsbury et al. 2006) and six loci in the yellow rail (*Coturnicops noveboracencis*, Miller et al. 2012).

Secondly, it is conceivable that genetic structure is indeed weak due to the relatively small spatial scale of this study combined with the dispersal ability of *C. malherbi.* North Canterbury may be too small for genetic differentiation to accumulate if *C. malherbi* are capable of dispersing among the Hawdon, Hurunui and Poulter valleys. For forest-dependent taxa such as *C. malherbi* the level of gene flow and therefore patterns of genetic diversity will depend on their ability to disperse through any surrounding unsuitable landscape. As the three North Canterbury valleys are situated within a 30 km radius and are connected by smaller patches of *Nothofagus* forest some level of dispersal among valleys is highly plausible (Kearvell pers. comm). Also as *C. malherbi* feed mainly on fruit and a range of both native and non-native vegetation (Ortiz-Catedral & Brunton 2009) it is conceivable that these birds forage in more open spaces at forest edges. Similar results were obtained for the forest-dependent passerine, the white-ruffed Manakin which inhabits nine forest fragments within an 18km radius within Costa Rica (Barnett et al. 2008). These authors concluded that the weak genetic structure (K = 1, and low to moderate F_{ST} values) indicated that these frugivorous birds are likely to forage in more open habitats and are therefore likely to move between forest patches. The persistence of species and populations within metapopulations depends on both the demographic connectivity and gene flow between patches (Harrisson et al. 2012). Dispersal however, is not necessarily accompanied by gene flow as migrants may have limited reproductive success (Harrisson et al. 2012). For example,

Peery et al. (2010) found that populations within a marbled murrelet (*Brachyramphus marmoraltus*) metapopulation remained genetically differentiated because migrants had less reproductive success than the resident breeding population. However, Haig et al. (2011) caution against making inferences about the connectivity within a metapopulation based on genetic data, citing previous research where field studies did not support genetic findings. Genetic structure does not describe the geographical boundaries of populations but can be used to make inferences about the movement of individuals when the undertaking of demographic studies is logistically difficult to carry out (Evanno et al. 2005; Stow & Magnusson 2012). Undertaking demographic studies of the cryptic and secretive *C. malherbi* occupying the high forest canopy of the North Canterbury high country are particularly challenging (Triggs & Daugherty 2006; Ortiz-Catedral & Brunton 2009; John Kearvell pers. comm.). Therefore, knowledge of genetic structure provides some insight into dispersal and population connectivity of these natural *C. malherbi* populations.

Tracy & Jamieson (2011) stress the importance of determining whether the current pattern of population structure reflects historical natural barriers to gene flow or latitudinal clines and is therefore of evolutionarily significance or reflects more recent human-mediated decline, isolation and fragmentation. Only contemporary *C. malherbi* samples were used in this study and therefore the processes underlining the observed structure are unknown. It is plausible that *C. malherbi* populations experienced similar contemporary range contractions, declines and fragmentation as other NZ endemic forest birds. For example, the endangered endemic forest passerine the NZ mohua (yellowhead, *Mohoua ochrocephala*) was once widespread throughout the South Island but is now restricted to forest remnants in North Canterbury and Southern regions of the South Island (Tracy & Jamieson 2011) . A

molecular study of both historical and contemporary mohua samples revealed current levels of genetic diversity and population structure were indicative of anthropogenic fragmentation and therefore had no evolutionary significance (Tracy & Jamieson 2011). Consequently, these authors recommended augmenting smaller populations with birds sourced from other regions as historically there was little genetic differentiation. Although my research did not include historical samples, past records of *C. malherbi* abundance and distribution plus the observed population declines during the 1999 and 2000 breeding seasons indicate that the current population structure conceivably reflects anthropogenic habitat fragmentation. Therefore, I recommend that existing captive and translocated populations are supplemented with birds sourced from the Poulter Valley.

Implications for conservation management

The genetic diversity and weak but significant population structure described in this study indicates that some level of connectivity exists among the three North Canterbury populations. Whether these results reflect a small sample size, recent fragmentation or historical structure is unknown but based on these findings I recommend managing the North Canterbury populations as one genetic population that is weakly subdivided into three semi-isolated sub-populations. Additionally, that the selection of and breeding between individuals from all three source populations within the captive breeding and translocated populations will conserve current levels of genetic diversity. The higher level of both mitochondrial and nuclear diversity detected in the Poulter Valley individuals indicates that birds sourced from this population will perhaps introduce additional genetic diversity into the current captive breeding and translocated populations. I also recommend the on-going genetic monitoring and management of captive and translocated populations to guide future conservation management of this critically endangered kākāriki.

Chapter 4: The genetic distinctiveness of sympatric *Cyanoramphus malherbi* & *C. auriceps* in North Canterbury

Abstract

The orange-fronted kākāriki (*Cyanoramphus malherbi*,) was once considered a colour-morph of the more abundant yellow-crowned kākāriki (*C. auriceps*). However, research into the taxonomic status of this New Zealand endemic over the past thirteen years based on morphological, behavioural, ecological and genetic analyses indicates that the orange-fronted kākāriki is a distinct species. Currently, sympatric critically endangered *C. malherbi* and near threatened *C. auriceps* populations occur in the Hawdon, Hurunui and Poulter valleys of North Canterbury. Despite strong evidence for assortative mating, a few mixed pairs have been observed following a recent drastic population decline in *C. malherbi* relative to *C.auriceps*. This study used both nuclear and mitochondrial sequence data, and microsatellite genotype data to determine the genetic distinctiveness of *C. malherbi* and *C. auriceps*. These data were also used to identify putative cryptic hybrids. Twenty one *C. malherbi*, ten *C. auriceps* and two putative hybrids from the now extinct Hope Valley were genotyped at 18 microsatellite loci and sequenced at one mitochondrial (cytochrome *b*) and one nuclear loci (RAG-1). The number of microsatellite alleles per locus ranged from 2 - 5 in *C. malherbi*, 2-4 in *C. auriceps* and 1-3 in the Hope Valley birds. Based on both cytochrome *b* and microsatellite markers, *C. malherbi* and *C.auriceps* were found to be genetically distinct (ϕ_{ST} = p<0.01; F_{ST} = 0.073, p < 0.01; K=2), and the two Hope Valley birds were confirmed to be hybrids. No diagnostic SNPs were found in either cytochrome *b* or RAG-1 sequences but both had variable sites producing three haplotypes and two alleles, respectively. These

findings lend support to the hypothesis that when one species is rare and the other abundant, limited hybridisation between sympatric populations of *C. malherbi* and *C. auriceps* is possible. Even though hybridisation is rare between these two species the prevalence may increase if *C. malherbi* numbers decrease. Therefore, current conservation strategies undertaken for *C. malherbi* such as the restoration and protection of mainland habitats, and increasing the number and size of populations through captive breeding and translocations are likely reduce the prevalence of hybridisation.

Introduction

The orange-fronted kākāriki (*Cyanoramphus malherbi*,) was once considered a colour-morph of the more abundant yellow-crowned parakeet (*C. auriceps*) (Taylor et al. 1986; Taylor 1998; Kearvell et al. 2003). The two species are morphologically similar but have a few distinguishing characteristics. *C. malherbi* have blue-green contour feathers, a narrow orange band above the beak, and an orange patch on each rump. In contrast *C. auriceps* have olive-yellow-green contour feathers, a pale-yellow fore-crown and a red rump patch (Birdlife International 2012) (Fig 1).

Fig 1: orange-fronted kākāriki (*C. malherbi*) (left) & yellow-crowned parakeet (*C. auriceps*) (right).

Photo: Department of Conservation

Previous research into the taxonomic status of the critically endangered *C. malherbi* over the past thirteen years based on morphological (Young & Kearvell, 2001), behavioural (Boon et al. 2000; Kearvell & Briskie 2003), ecological (Kearvell et al. 2002), and genetic (Boon et al. 2000) analyses indicate that the orange-fronted kākāriki is a distinct species. Currently, *C. malherbi* is restricted to a 360km^2 area in the Hawdon, Hurunui and Poulter valleys of North Canterbury, New Zealand where they occur sympatrically with *C. auriceps* (Kearvell et al. 2002; Birdlife international 2012). The Hurunui population of *C. malherbi* experienced a substantial population decline in response to a rat irruption following two consecutive beech mast years in 1999 and 2000 (Birdlife International 2012). As a result, *C.malherbi* numbers fell from an estimated population of 500 - 700 to approximately 200 in these valleys (Birdlife International 2012). Historically, *C. auriceps* has been observed in greater numbers than *C. malherbi* within sympatric mainland populations (Taylor et al. 1986; Kearvell et al. 2003). For example, Kearvell et al. (2003) quotes the naturalist Thomas Potts in 1885 as saying *C. malherbi* were "nowhere as common" as yellow-crowned or red-crowned parakeets (*C. novaezelandiae)* and Taylor et al. (1986) recorded a ratio of one *C. malherbi* to 12 *C. auriceps* within the Lake Sumner Forest Park in 1980. Declines in the abundance and distribution of mainland parakeets since European settlement have been attributed to habitat modification, predation by introduced mammals and competition for resources from both native and introduced species (Kearvell et al. 2002; Birdlife International 2012).

Habitat modification alters species' abundance and distribution by fragmenting landscapes into small, isolated patches (Crispo et al. 2011; Harrisson et al. 2012). Unsuitable habitats

between patches create barriers for dispersal for some species, reducing gene flow and increasing the genetic differentiation among these populations (Crispo et al. 2011; Harrisson et al. 2012). Simultaneously, modified habitats provide opportunities for contact and gene flow among previously isolated populations and species, increasing the likelihood of hybridisation and decreasing the genetic differentiation among these populations (Crispo et al. 2011). Interspecific hybridisation is widespread among birds and can occur in response to these changes in both species' abundance and distribution and is of conservation concern when the existence of rare and /or endemic species is threatened (Triggs & Daugherty 1996; Chan et al. 2006; Väli et al. 2010; Crispo et al. 2011) Interspecific hybridisation is also known to occur in response to differences in the relative abundance of closely related species (Randler 2006; Crispo et al. 2011). Consequently, individuals of the rarer species switch to heterospecific mates due to the scarcity of conspecific mates (Randler 2006; Crispo et al. 2011). A shortage of conspecifics is thought to have facilitated hybridisation between yellow and red- crowned parakeets on Little Barrier Island resulting in viable hybrid offspring (Triggs & Daugherty 1996). Hybridisation between Forbes' (*C. forbesi*) and the Chatham Island red-crowned (*C. novaezelandiae chathamensis*) parakeets on Little Mangere and Mangere Islands (Chatham Island group) was first observed in the 1970s (Taylor 1975; Triggs & Daugherty 1996). Extensive deforestation had by 1969 restricted *C. forbesi* to "about six hectares of forest and scrub" (Taylor 1975) and predation by cats resulted in a substantial population decline of Forbes' parakeet (Triggs & Daugherty 1996). Subsequent immigration and colonisation by the Chatham Island red-crowned parakeets into the resultant open habitats is thought to have promoted the hybridisation between these two species (Triggs & Daugherty 1996; Chan et al. 2006). Although *C. malherbi* have generally been considered the rarest of the mainland *Cyanoramphus* parakeets, the contemporary population declines in

the Hurunui valley are of particular concern as hybridisation between sympatric *C. malherbi* and *C. auriceps* was suspected in the now extinct Hope Valley when the *C. malherbi* population was extremely small (Kearvell et al. 2003, Fig 2).

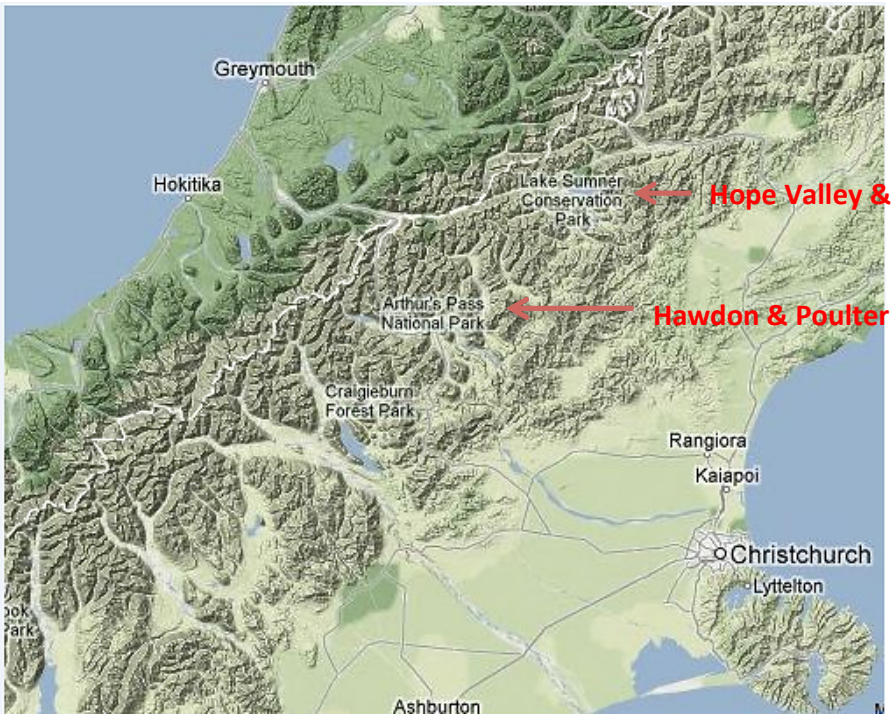

Fig 2: Location of the South branch of the Hurunui plus the Hope, Hawdon, & Poulter Valleys within North Canterbury. Google Maps.

Subsequent mitochondrial control region sequence data confirmed that two Hope Valley birds (WG612 & IS1) morphologically identified as *C. malherbi* were cryptic hybrids as both were found to have *C. auriceps* haplotypes (Boon et al. 2000). Another Hope Valley bird (WG611) also identified as *C. malherbi* was found to have a *C. malherbi* haplotype but because mitochondrial DNA is maternally inherited it may also be cryptic hybrid. Despite the presence of apparent cryptic hybrids, observational evidence suggests hybridisation is likely to be rare. For example, field studies to detect the presence of *C. malherbi* and *C. auriceps* mixed pairs were undertaken in the South Branch of the Hurunui between 1996 and 1999

but no mixed pairs were encountered and the two species were observed to pair assortatively (Boon et al. 2000). Recently, a more comprehensive study provides definitive evidence for strong assortative mating between *C. malherbi* and *C. auriceps* within the three North Canterbury populations as only 1% of mixed breeding pairs were found despite relatively low numbers of *C. malherbi* (Kearvell & Steeves, in preparation).

This study used both nuclear and mitochondrial DNA sequence and microsatellite genotype data to determine the genetic distinctiveness of *C. malherbi* and *C. auriceps* and to identify putative cryptic hybrids within sympatric populations of these two species. As mitochondrial DNA only provides information about maternal lineages it was important to also include biparentally inherited nuclear markers such as microsatellites to provide information about both maternal and paternal lineages in order to discriminate between species and estimate levels of hybridisation and introgression (Pacheco et al. 2002; Edwards and Bensch 2009; Väli et al. 2010).

Materials and Methods

Sampling

C. auriceps samples were collected during the 2010 / 2011 breeding season from the Hawdon and Hurunui valleys by members of the orange-fronted kākāriki recovery group. Blood and tissue samples previously collected from wild *C. auriceps* in the Hurunui and Hawdon valleys in 1996 were also obtained from Victoria University, Wellington. Please refer to chapter 3 regarding the collection of *C. malherbi* samples from the Hawdon, Hurunui and Poulter valleys. In addition, three *C. malherbi* samples that had mixed Hawdon / Hurunui parentage were also obtained from the captive population at Peacock Springs. The Hope Valley tissue samples were obtained from Victoria University but the date of

collection is unknown. A total of 21 *C. malherbi*, ten *C.auriceps* and two Hope Valley individuals were used in this study.

Given the importance of only including unrelated birds of known provenance, *C. malherbi* samples sourced from Peacock Springs were considered unrelated if they did not share grandparents. For wild samples, both *C. malherbi* and *C. auriceps* individuals were considered unrelated if they originated from different nests. No *C. auriceps* samples from the Poulter valley were available (refer to Appendix 1.for a detailed list of samples). All samples are now stored permanently at the School of Biological Sciences, University of Canterbury.

DNA extraction

The DNA extracts from the previous chapters were included in this study and additional samples were extracted using the same methods (see Chapter 3).

Microsatellites

All samples were genotyped at the 17 polymorphic microsatellite loci developed specifically for this study. The relevant methodology is outlined in Chapter two. In addition, one microsatellite locus (Cfor 0809) developed for Forbes' parakeet (*C. forbesi*) and successfully amplified in *C.malherbi* and *C.auriceps* by Chan et al. (2005) was screened on all samples using the same PCR protocol described in Chan et al. (2005). All reactions were run in a Mastercycler EP thermocycler (Eppendorf), and PCR products were visualised by electrophoresis on a 2% agarose gel prestained with SYBRsafe (Invitrogen). Allele sizes were determined using the size standard Genescan® 500-LIZ (Applied Biosystems), and scored manually using GENEMARKER (version 1.9; SoftGenetics).

Estimates of genetic diversity, tests for significant deviations from Hardy-Weinberg equilibrium, tests for significant linkage disequilibrium and estimates of population structure (F_{ST}) were calculated using ARLEQUIN (version 3.5; Excoffier & Lischer 2010). F'_{ST} was also calculated to correct for within-population diversity (Meirmans & Hedrik 2011). Multiple pairwise comparisons were corrected using the Benjamini and Yekutieli (B-Y) correction (Narum 2006). To test whether *C. malherbi* and *C.auriceps* are genetically distinct, I used the Bayesian clustering algorithm implemented in STRUCTURE (version 2.3; Pritchard et al. 2010). Ten independent replicates (20,000 burn-in and 200,000 steps) with different random seeds were run for K = 1-4 using the admixture ancestry model, correlated allele frequency model and sampling location as a prior as per the recommendations by Hubisz et al. (2009) for small sample sizes and weak genetic structure. I determined the number of clusters (K) by comparing corrected estimates of the posterior probability, denoted $L(K)$, for each value of K using a likelihood ratio test. To detect cryptic hybrids in the Hope Valley samples, this analysis was repeated using the Hurunui *C. malherbi* (n = 6) and *C.auriceps* (n = 5) samples due to the close proximity of these two valleys. This also gave me the largest number of samples from both species. The population assignment method implemented in STRUCTURE was also used to detect inter-specific hybrids (Pritchard et al. 2010). This Bayesian clustering approach estimates the level of admixture (q), calculated as the proportion of the genome for each individual that is derived from the two parental species. I set a q-value of 0.20 as a significant threshold to identify hybrids and parental species as this threshold has been shown to give the best performance in respect to hybrid identification accuracy (the proportion of purebred and hybrid individuals that actually belong to that category) and efficiency (the proportion of purebred and hybrid individuals in a group that were correctly identified) (Väli et al. 2010; Vähä and Primmer 2006).Therefore,

any individual that derived more than 80% of their genome (0.20 ≥ q ≥ 0.80) from either *C. malherbi* or *C. auriceps* was assigned to that species. This conservative q value also reflects my small sample size as allele frequencies calculated will have a large error rate. Individuals with intermediate q-values (0.2 ≤ q ≤ 0.80) were classified as hybrids and first (F1) generation individuals those with a q-value of 0.5 (Vähä and Primmer 2006).

A Principle Components Analysis (PCA) to further visualise the pattern of genetic differentiation were performed using GENALEX (version 6.4.1; Peakall & Smouse 2006).

SNPs

Previously published DNA sequences from the mitochondrial cytochrome *b* and the nuclear exons RAG-1 and CMOS genes stored in Genebank were aligned in GENEIOUS (version 5.5.4; Biomatters) to locate putative species-specific (diagnostic) and polymorphic sites. The primers used to target these sites plus the amplification, screening, sequencing and genotyping of both mitochondrial and nuclear SNPs are described in detail in Chapter 3. SNP diversity for each gene and each species was calculated using ARLEQUIN (version 3.5; Excoffier & Lischer 2010). The RAG-1 data was also combined with the microsatellite data to determine population structure in ARLEQUIN and for both population and assignment tests in STRUCTURE.

Results

Microsatellites

Deviations from Hardy-Weinberg, significant linkage disequilibrium, null alleles and chromosomal location and missing data for all loci and both species are reported in Chapter two. Allelic diversity in *C. malherbi* ranged from 2 -5; *C. auriceps* 2-4 and the Hope Valley samples 1-3 (refer Chapter 2). F_{ST} for samples pooled across all valleys showed that *C. malherbi* and *C.auriceps* individuals were significantly different (F_{ST} = 0.073, F'_{ST} = 0.12; p <

0.01, Table 1). The results were similar when microsatellite and RAG-1 data were combined (F_{ST} = 0.071, p < 0.01; Table 1). Significant genetic differentiation was also found between Hurunui *C. malherbi* and *C.auriceps* individuals (F_{ST} = 0.14, p < 0.01, Table 1). Again the results were similar when only microsatellites and when RAG-1 data was included (F_{ST} = 0.15, p < 0.01; Table 1).

Table 1: *C. malherbi* & *C. auriceps* overall F_{ST} and F_{ST} for the Hurunui only		
	Global F_{ST}	Hurunui
Microsatellites	**0.073 (p < 0.01)**	**0.14 (p < 0.01)**
Microsatellites & RAG-1	**0.071 (p < 0.01)**	**0.15 (p < 0.01)**

Highlighted figures are significant (p < 0.05)

STRUCTURE analysis of both microsatellite data and combined microsatellite and RAG-1 data provided evidence of genetic structure. Among the levels of K investigated for both species (Fig 1), the highest average likelihood score was significantly higher for K = 2 than K = 1 (likelihood ratio = 24.02, p < 0.0001). These data combined with an alpha value close to zero indicates that the appropriate number of clusters is two. Among the levels of K investigated for the Hurunui (Fig 2) the highest average likelihood was also significantly higher for K = 2 than K = 1 (likelihood ratio = 7.78, p < 0.05). For the assignment test, *C. malherbi* and most of the *C.auriceps* individuals had an assignment probability of ≥0.80 to clusters one and two respectively (Fig 3). One *C. auriceps* individual was borderline (FT3311, q = 0.21). The two Hope Valley birds were assigned to the *C. auriceps* cluster with q values of 0.39 (WG612) and 0.25 (WG611), confirming their admixed ancestry (Fig 3). No individuals were found to have intermediate q-values suggesting that no first (F1) generation hybrids were identified.

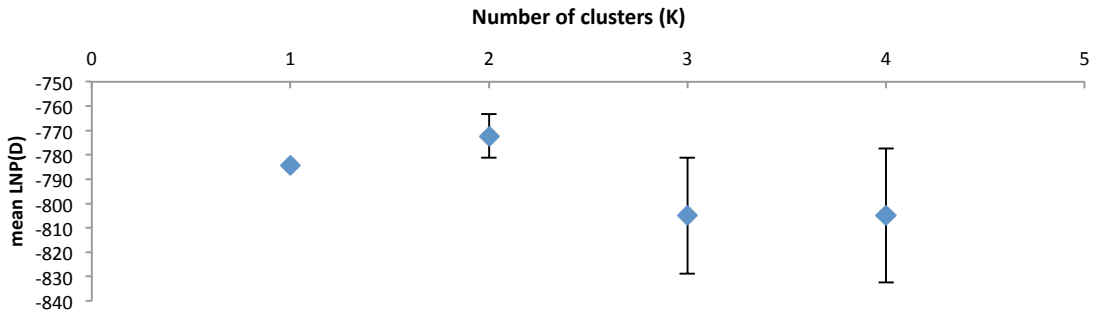

Fig 1: Determination of the most likely number of clusters (K) between all *C. malherbi* & *C. auriceps* samples based on microsatellite data. Plot of mean (± SD) log likelihoods of STRUCTURE analysis for K= 1 - K = 4

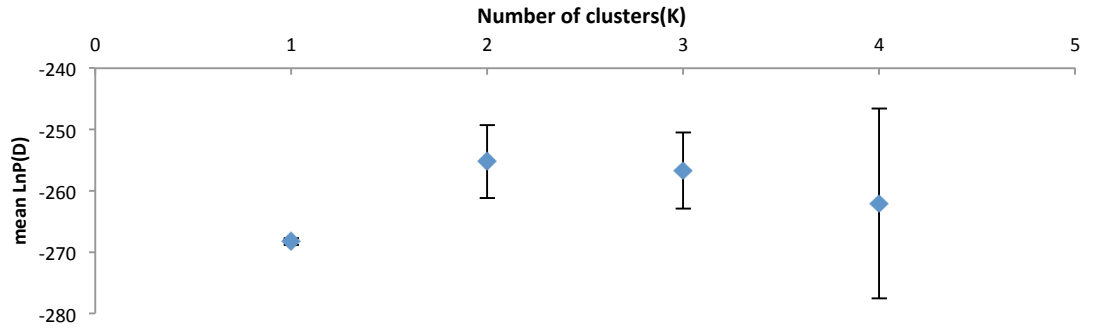

Fig 2: Determination of the most likely number of clusters (K) between Hurunui *C. malherbi* & *C. auriceps* based on microsatelitte data.
Plot of mean (± SD) log likelihoods of STRUCTURE analysis for K = 1 - K = 4

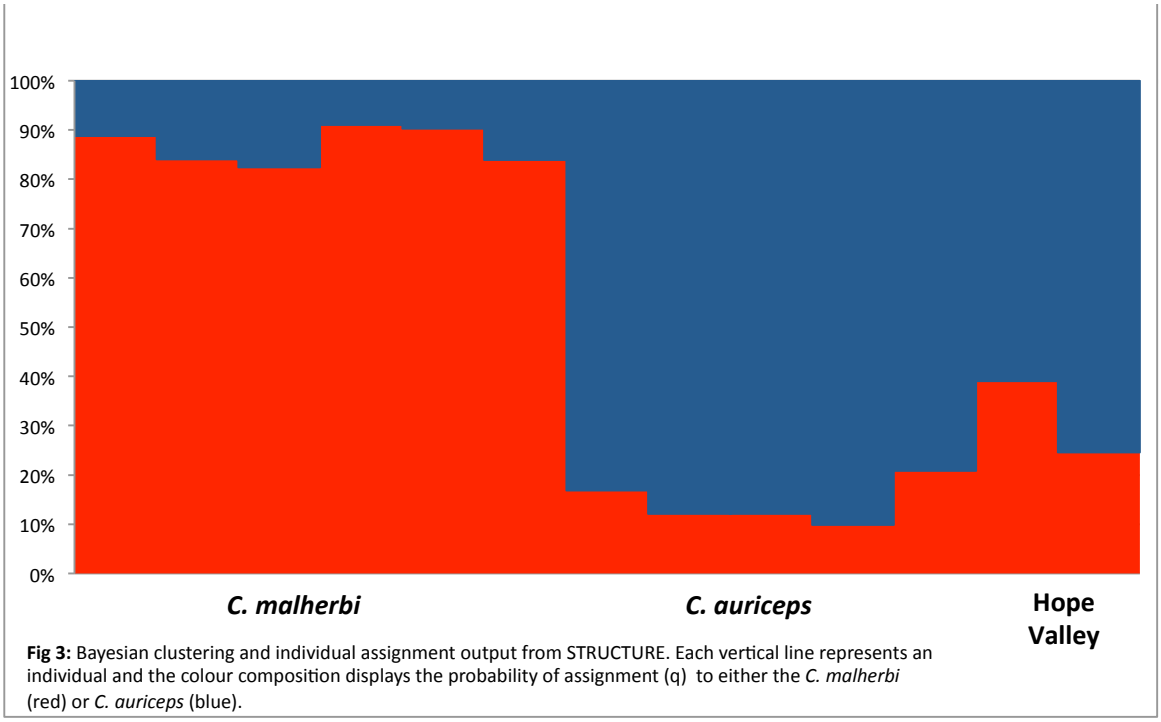

Fig 3: Bayesian clustering and individual assignment output from STRUCTURE. Each vertical line represents an individual and the colour composition displays the probability of assignment (q) to either the *C. malherbi* (red) or *C. auriceps* (blue).

The principal component analysis (PCA) clustered *C. malherbi* and *C. auriceps* with some separation with the exception of two *C. malherbi* (SB280 & C001) and two *C. auriceps* individuals (FT3311 & YCP7, Fig 4). The PCA of Hurunui *C. malherbi* and *C. auriceps* individuals clustered the two species and the two Hope valley birds separately (Fig 5). The results were similar when microsatellite and RAG-1 data were combined (Appendix 3).

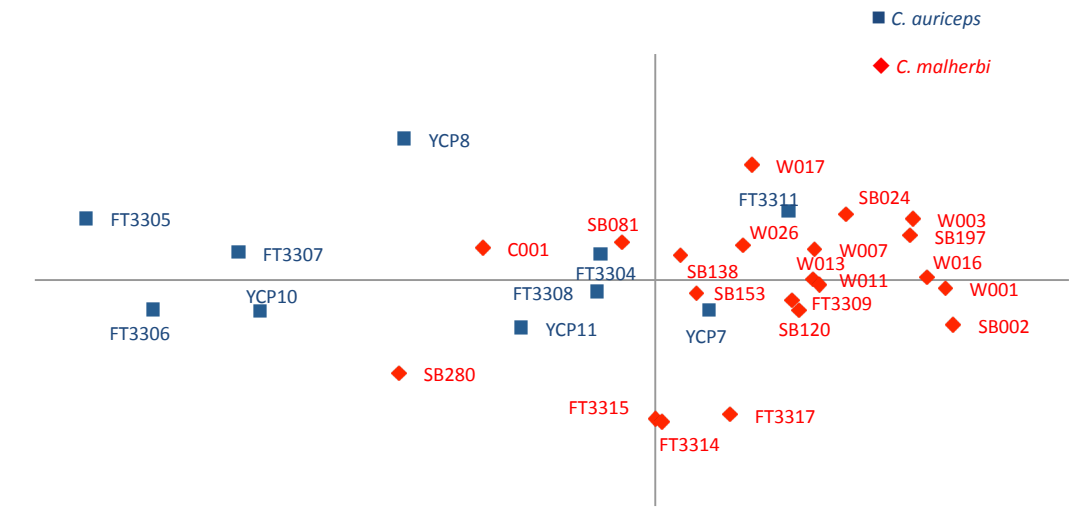

Fig 4: Principle components analysis (PCA) of multilocus genotypes based on microsatellite data for all *C. malherbi* & *C. auriceps* samples.

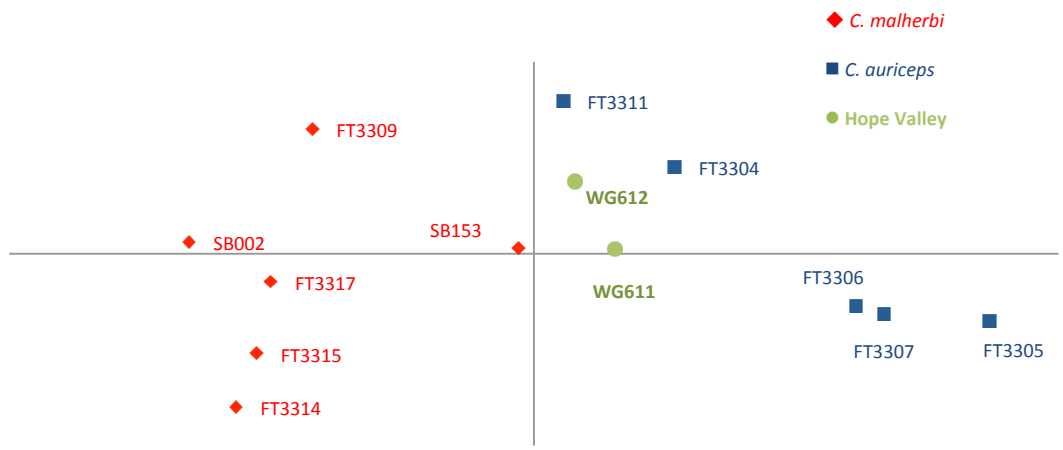

Fig 5: PCA of multilocus genotypes based on microsatellite data of all Hurunui *C. malherbi* & *C. auriecps* and Hope Valley individuals based on microsatellites genotypes

Mitochondrial cytochrome *b*

Twenty *C. malherbi*, ten *C.auriceps* and the two Hope Valley samples amplified and sequenced successfully. No diagnostic SNPs were found but four variable sites were found at positions 80, 154, 340 and 469 (C↔T) producing three haplotypes (A, B & C). Haplotypes A, B and C were found in all *C. malherbi*; but only haplotypes (A & B) were found in *C.auriceps* (Table 2). One Hope valley sample (WG611) had haplotype C and the other

Table 2: Distribution and frequencies of Cytochrome *b* haplotypes in *C. malherbi, C. auriceps* & Hope Valley individuals

Cyt*b* Haplotype	Site				*C. malherbi*	*C. auriceps*	Hope Valley
	80	154	340	469			
A	C	C	C	C	6	9	0
B	•	T	•	•	1	1	1
C	T	T	T	T	14	0	1
				Total	21	10	2

Table 3: RAG-1 genotypes & frequencies for *C. malherbi, C. auriceps* & Hope Valley individuals

RAG-1 Genotypes	Site	*C. malherbi*	*C. auriceps*	Hope Valley
	235			
CC		11	8	2
CT		5	0	0
TT		2	1	0
	Total	18	9	2

(WG612) haplotype B (Table 3). Estimates of global Φ_{ST} indicated significant genetic structure between *C. malherbi* and *C. auriceps* (Φ_{ST} = 0.53, p < 0.01). Significant genetic differentiation between the two species was also detected within the Hurunui populations (Φ_{ST} = 0.75, p = 0.04).

Nuclear RAG-1

Only two samples (one *C. malherbi* and one *C.auriceps*) failed to amplify. No diagnostic SNPs were located. Only one variable site was found at position 235 (C↔T). All *C.auriceps* and both Hope Valley samples were homozygous with either CC or TT at this site, whereas five *C. malherbi* were heterozygous and 15 homozygous (Table 3). Estimates of global F_{ST} values indicated no significant structure between *C. malherbi* and *C.auriceps* (F_{ST} = 0.018, p = 0.32) or between the two species in the Hurunui (F_{ST} = 0.013, p = 0.25).

Nuclear C-MOS

Despite attempting to optimise amplification conditions the majority of samples failed to amplify reliably so the decision was made to exclude C-MOS from this study.

Discussion

This study used mitochondrial and nuclear sequence and microsatellite genotype data to determine the genetic distinctiveness of *C. malherbi* and *C. auriceps* and to identify putative cryptic hybrids. The low estimates of allelic diversity (*C. malherbi* 2 – 5; *C. auriceps* 2 – 4 alleles / locus) in this study are consistent with other threatened and endangered species as outlined in Chapter three. The sample sizes used in this study were less than optimal and therefore the overall allelic diversity of the each population may have been underestimated (Nazareno & Jump 2012). Although very rare alleles (frequencies of < 0.01) are unlikely to have been sampled, this is possible even if I had an optimal sample size of 25 – 30

individuals per population (Hale et al. 2012). Therefore, whilst a sample size of 21 *C. malherbi* and ten *C. auriceps* may not have detected all the alleles present in a population, the use of 18 microsatellite loci this study is likely to have achieved a reasonable estimate of genetic diversity and allele frequencies (Hale et al. 2012; Landguth et al. 2012; Struebig et al. 2012).

The biparentally inherited microsatellite genotype data and uniparentally inherited mitochondrial cytochrome *b* sequence data obtained in this study supports previous mitochondrial control region sequence data analysed by Boon et al. (2000) that *C.malherbi* and *C.auriceps* are indeed genetically distinct (Tables 1 & 2). No significant genetic differences were detected by the RAG-1 SNP data, which can be attributed to the relatively slow mutation rate of exons (10^{-8} - 10-^{9}) compared to microsatellites (10^{-3} – 10^{-4}) (Paton & Baker 2006). In addition, mutation rates differ by an order of magnitude between nuclear exons and mitochondrial genes (10^{-7} –10^{-8}) but exons are biparentally inherited and the effective population size (N_e) for nuclear loci are four times that of uniparentally inherited mitochondrial loci (Zink and Barrowclough 2008). Exons such as the RAG-1 gene used in phylogenetic studies are not always useful for distinguishing between species living in sympatry as both alleles of biallelic SNPs may occur at high frequencies in both species (Väli et al. 2010). The low mutation rate associated with exons make these markers ideal for phylogenetic studies but the higher levels of variation associated with introns suggests these may be a better source of species-specific SNPs for studies of hybridisation and introgression in closely related species (Pacheco et al. 2002). I therefore suggest that SNPs are developed from introns for future population genetic studies of *Cyanoramphus* parakeets.

Based on the microsatellite data, sympatric *C. malherbi* and *C.auriceps* individuals in the Hurunui are significantly different from each other (F_{ST} = 0.14, p < 0.01; Table 1). These results also support the field observations of assortative pairing carried out in the Hurunui by Boon et al. (2000) and assortative mating within the three North Canterbury populations by Kearvell & Steeves (in preparation). My research confirmed that the Hope Valley bird (WG612) with a *C.auriceps* haplotype (Boon et al. 2000) is a *C. malherbi* x *C.auriceps* hybrid with an assignment to the *C.auriceps* cluster of q = 0.39. I also established that the other Hope Valley bird (WG611) found to have a *C.malherbi* haplotype by Boon et al. (2000) was also a hybrid with an assignment to the *C.auriceps* cluster of q = 0.25. As neither of these two birds were found to be first generation (F1) hybrids (q = 0.50) it seems likely that F1 individuals are fertile and therefore introgression is possible. These two birds are possibly the product of mating between two hybrids or a hybrid backcrossing with a parental species but I do not have the data to support either of these scenarios. As hybridisation between these two species is likely to be rare, it is probable that F1 offspring can only backcross with a parental species (Senn & Pemberton 2009).

Hybridisation between sympatric populations of closely related species is not uncommon in birds especially when one species is less common and conspecific mates are unavailable (Randler 2006; Simeone et al. 2009). For example, restricted mate choice is thought to have facilitated hybridisation between sympatric populations of Humboldt (*Spheniscus humboldti*) and Magellanic (*S. magellanicus*) penguins on islands off the coast of Chile. However, assortative mating occurred in populations where the abundance of both penguin species was similar (Simeone et al. 2009). Similarly, despite strong assortative mating the critically endangered endemic black stilt (*Himantopus novaezelandiae*) and the more

abundant self-introduced pied stilt (*H. himantopus leucoephalus*) are known to hybridise due to lack of conspecific mates (Steeves et al. 2010). In the Falkland Islands, the abundant speckled teal (*Anas flavirostris*) outnumbers the yellow-billed pintail (*A. georgica*) by ten to one. McCracken & Wilson (2007) found that interspecific hybridisation occurred on these islands but not within mainland Argentinian populations where both species are equally abundant. Previous research suggests that when conspecific mates are rare, *Cyanoramphus* species are able to hybridise as observed on Mangere Island (*C. forbesi* x *C. novaezelandiae chathamensis*) and Auckland Islands (*C.auriceps* x *C. novaezelandiae*) but that assortative mating inhibits interspecific hybridisation when conspecific mates are available (Taylor 1975; Triggs & Daugherty 1996; Chan et al. 2006). As hybridisation events are likely to be rare and there is only one year when *C. malherbi* is known to have been as common as *C. auriceps* (Kearvell & Steeves in preparation), I do not have the sample size nor data to conclude whether hybridisation between *C. malherbi* and *C. auriceps* in the Hope Valley occurred in response to reduced *C. malherbi* numbers but when one species is rare and the other abundant, limited hybridisation between sympatric populations of these two species is possible. Habitat modification and reduction in *C. malherbi* population size conceivably have facilitated the hybridization between these two species.

Even though hybridisation is rare between these two species the prevalence may increase if *C. malherbi* numbers decrease. Current conservation strategies undertaken for *C.malherbi* such as the restoration and protection of mainland habitats plus increasing the number and size of populations through captive breeding and translocations may reduce the prevalence of hybridization. Currently, sympatric populations of *C. auriceps* and translocated *C. malherbi* occur on Chalky and Maud Islands but *C. auriceps* is absent from Tehua and

Blumine Islands (Elkington & Kearvell, 2010). As future natural migration to these islands by *C. auriceps* is possible (Elkington & Kearvell, 2010) some level of hybridisation in the future is conceivable. It is important therefore, to determine the degree of threat that hybridisation poses for *C.malherbi*. For example, some degree of hybridisation may increase genetic diversity plus fitness in modified environments and therefore greater evolutionary potential and persistence (de Woody et al. 2010).

Implications for Conservation

The nuclear and mitochondrial genetic markers developed in this study will provide a useful and efficient molecular genetic resource for the identification of both pure and admixed *C.malherbi* and *C. auriceps* individuals. This study has demonstrated that genetically admixed individuals are difficult to identify morphologically. Consequently, the accurate identification and monitoring of *C. malherbi* in the field is particularly difficult and therefore reinforces the need for the thorough identification protocol based on the plumage colour of either the frontal band or rump patches developed by the orange–fronted kākāriki recovery group (Kearvell & Steeves in preparation). To ensure the genetic integrity of *C. malherbi* it is essential to reduce the risk of introducing cryptic hybrids into the captive and translocation populations I therefore, strongly recommend the use of these molecular methods to accurately identify all individuals prior to entering the captive breeding and translocation programmes.

Chapter 5: Discussion

Over the past two decades a broad range of research has increased our knowledge and understanding of *Cyanoramphus* parakeets. For example, taxonomy and molecular systematics (Triggs & Daugherty 1996; Boon et al. 2000), ecology (Greene 1998; Kearvell et al. 2002), behaviour (Kearvell & Briskie 2003; Ortiz-Catedral & Brunton 2009), hybridisation (Chan et al 2006), sexing (Tokunaga et al. 2007), biogeography (Chambers et al. 2001; Holdaway et al. 2010) and the identification and screening of disease (Massaro et al. 2012; Sikorski et al. 2013;). My thesis adds to this knowledge by providing species-specific information on the critically endangered orange-fronted kākāriki (*C. malherbi*) at a local scale. In addition, my research has provided useful genetic tools and information to inform active conservation management of captive and translocated populations of *C. malherbi.* To achieve this, my research had the following objectives:

The first objective was to develop microsatellites for *C. malherbi* using next-generation sequencing technology. I chose to use high throughput genomic sequencing as microsatellites are thought to occur at low frequency in avian genomes (Abdelkrim et al. 2009; Miller et al. 2013) and 454-sequencing has been shown to produce more loci than previous methods (Gardner et al. 2011). Prior to high throughput sequencing many avian studies used between five and ten microsatellite loci (Haig et al. 2011). The eighteen polymorphic loci I obtained from 16,497 reads are greater than similar studies in birds which also used high-throughput sequencing. For example, Abdelkrim et al. (2009) developed 13 polymorphic loci from 17,215 reads in the New Zealand endangered blue duck (*Hymenolaimus malacorhynchos*). Stoeckle et al. (2012) developed 12 polymorphic loci from 52,583 reads in the endangered New Caledonian Kagu (*Rhynochetos jubatus*) and Miller et

al. (2013) 14 loci from 73, 522 reads in the Tasmanian orange-bellied parrot (*Neophema chrysogaster*).

The second objective was to combine nuclear and mitochondrial sequence data with microsatellite genotype data to quantify genetic diversity within and population genetic structure among the Hurunui, Poulter and Hawdon populations of *C. malherbi.* Subsequent analyses of these data were used to ascertain the suitability of including Poulter Valley birds in the captive breeding and translocation populations. My analyses demonstrated that the estimates of allelic diversity and expected heterozygosity in *C. malherbi* were consistent with other threatened bird species. Also, that there some level of connectivity exists among the three remaining natural populations of *C. malherbi*. Hence, I concluded that the three valleys function as a metapopulation.

The third objective was to use these nuclear and mitochondrial markers to confirm the genetic distinctiveness of *C. malherbi* and *C. auriceps* and identify cryptic hybrids within sympatric populations of these two species. Based on the microsatellite and cytochrome *b* sequence data, I concluded that *C. malherbi* and *C. auriceps* were genetically distinct and that two putative hybrid individuals were indeed cryptic hybrids. These findings lend support to the hypothesis that when one species is rare and the other abundant, limited hybridisation between sympatric populations of *C. malherbi* and *C. auriceps* occurs. My conclusions substantiate previous research into the taxonomic status of *C. malherbi* based on mitochondrial control region sequence data analysed by Boon et al. (2000). In addition, these results also support the field observations of assortative pairing carried out in the Hurunui by Boon et al. (2000) and assortative mating within the three North Canterbury populations by Kearvell & Steeves (in preparation).

The fourth objective was to provide useful and relevant genetic information to assist in the conservation management of wild, captive and translocated populations of *C. malherbi*. While genetic data doesn't always reflect demographic data, when good demographic studies are logistically difficult to achieve in cryptic species, genetic data can be used to make inferences about the demographic connectivity among populations (Evanno et al. 2005; Stow & Magnusson 2012). Based on my conclusions, I recommend the inclusion of individuals from each of the three source populations within the captive breeding and translocated populations to conserve current levels of genetic diversity (Chapter 3). In addition, to ensure the genetic integrity of *C. malherbi* I strongly recommend the use of these molecular methods to accurately identify all individuals prior to entering the captive breeding and translocation programmes (Chapter 4). I also recommend the on-going genetic monitoring and management of captive and translocated populations to guide future conservation management of this critically endangered kākāriki.

Future opportunities for research

Despite the sampling limitations, I have successfully achieved my objectives and provided relevant and practical recommendations for conservation management. The genetic markers presented here, particularly the microsatellites, would be informative for the following future research opportunities.

1. The preservation of current levels of genetic diversity and minimisation of inbreeding within *C. malherbi* captive populations. The molecular markers used in my research would enable the genetic relationships among the current captive population to be determined and the genotyping of additional individuals prior to their entering the captive breeding programme. Hagen et al. (2011) for example, used microsatellite data to investigate the relationship between genetic relatedness and reproductive success among kakī (black stilt, *Himantopus novaezelandiae*) individuals within the captive breeding population. Consequently, to minimise inbreeding depression within this small captive population, these authors were able to suggest guidelines for pairing of unrelated individuals.
2. The detection and monitoring of hybridisation between *C. malherbi* and *C. auriceps* within translocated or historical sympatric populations to determine the level of threat of hybridisation and to guide the selection of additional individuals and sites for future translocations. Steeves et al. (2010) for example, used microsatellite and mitochondrial sequence data to measure the levels of hybridisation between endemic kakī (black stilt, and the self-introduced pied stilt (poaka, *H. himantopus leucoephalus*). This study detected no evidence of introgression despite extensive bidirectional hybridisation between these two species.

3. The combining of historical and contemporary sampling to investigate the changes in genetic diversity and population structure overtime to assess the genetic impacts of anthropogenic habitat fragmentation. For example, Tracy & Jamieson (2011) using historical and contemporary samples, demonstrated that contemporary patterns of genetic structure in the New Zealand mohua (yellowhead, *Mohoua ochrocephala*) resulted from recent anthropogenic fragmentation. As current genetic structure had no evolutionary significance these authors recommended that fragmented mohua populations are managed as one genetic population.

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Appendix 1

Details of available samples, including family relationships

Abbreviations: SB (stud book number); W (collected dead from the wild); C (dead offspring from Peacock Springs)

C. malherbi samples collected from Poulter Valley (No captive bred samples available)

Source & GPS coordinates	Identification	Sample Type	Date Collected	Notes
Nest A	W003	Embryo	2008	Used in study
	W004	Embryo		
	W005	Embryo		
	W006	Embryo		
Nest 1 E1507073-N249081	SB192	Feathers	2008/2009	
	SB195	Feathers		
	SB196	Feathers		
	SB197	Feathers		Used in study
	SB198	Feathers		
	W014	Feathers		
Nest 5 E1508298-N5247566				
	W007	Unhatched Egg		Used in study
	W008	Unhatched Egg		
	W009	Unhatched Egg		
	W010	Unhatched Egg		
Nest 7 E1506578-N5249445				
	W013	Embryo		Used in study
	W015	Feather		
	W016	Embryo		
Nest 8 E1506704-N5248853	W017	Embryo		Used in study

	SB002	Whole bird	March 2003	Used in study
	SB011	Feathers	Dec 2003	Used in study
Nest 2	SB150	Feathers	2007/2008	
	SB151	Feathers		
	SB152	Feathers		
	SB153	Feathers		Used in study
From Victoria University	FT3309	Blood/Tissue	1996	Used in study
	FT3314	Blood/Tissue	1996	Used in study
	FT3315	Blood/Tissue	1996	Used in study
	FT3316	Blood/Tissue	1996	Excluded from data analysis as 45% missing data (failed to amplify)
	FT3317	Blood/Tissue	1996	Used in study
***C. malherbi* samples collected from the Hawdon Valley or captive bred from Hawdon parents.**				
Unknown	W001	Feathers & tissue	2007/2008	Used in study
E1498973-N5243734	W011	Feathers	2010	Used in study
	SB024	Feathers	2004	Used in study
	SB081	Whole Bird	2006	Used in study
Nest 4	SB280	Feathers	2010/2011	Used in study
	SB281	Feathers		
Nest 10	W022	Feathers	2010/2011	
E1497539-N5242276				
Nest 15	W024	Embryo	2010/2011	
E1497386-N5242381	W025	Embryo		
	W026	Embryo		Used in study
***C. malherbi* samples received from Peacock Springs (Family Tree page 96, illustrates the relationships among these individuals)**				
Mixed Hawdon/ Hurunui Parentage				
Offspring of SB016 (Hawdon) x SB011 (Hurunui)				
	SB032	Feathers		
	SB044	Feathers		
	SB121	Feathers		
	SB138	Whole bird		Used in Study (Chapter 3)

	SB139	Feathers		
	SB140	Feathers		
	SB141	Feathers		
	SB142	Feathers		
	SB157	Feathers		
Offspring of SB032 (HxH) x SB012 (Hawdon				
	SB108	Feathers		
	SB109	Feathers		
	SB111	Feathers		
	SB117	Feathers		
	SB120	Whole bird		Used in Study (Chapter 3)
	SB145	Feathers		
	SB146	Feathers		
	SB147	Feathers		
	SB148	Feathers		
	SB215	Feathers		
	SB213	Feathers		
	SB214	Feathers		
	SB216	Feathers		
	SB217	Feathers		
	SB218	Feathers		
	SB226	Feathers		
	SB227	Feathers		
Offspring of SB044 (HxH) x SB033 (Hawdon)				
	SB106	Feathers		
	SB107	Feathers		
	SB133	Feathers		
	SB134	Feathers		
	C001	Embryo		Used in Study (Chapter 3)
	C002	Embryo		
	C003	Embryo		
	C004	Embryo		

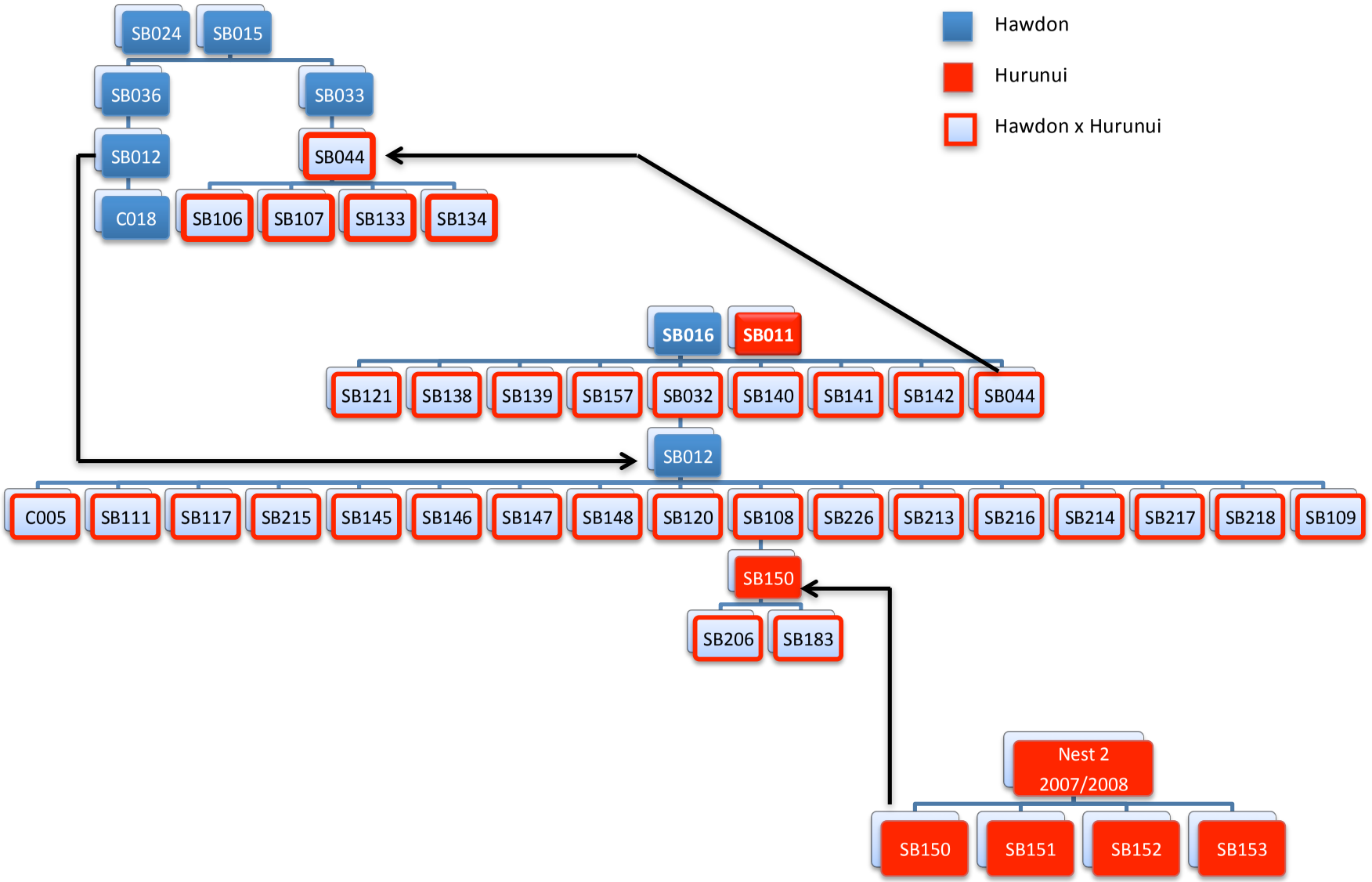

Fig1: Family tree illustrating the relationships of *C. malherbi* samples received from Peacock Springs

C. auriceps & Hope Valley samples

Source & GPS coordinates	Identification	Sample Type	Date Collected	Notes
Hawdon				
E1497533-N5243541	YCP 7	Feathers	2011	
E1497533-N5243541	YCP 8	Feathers	2011	
unknown	YCP 9	Feathers	2010	Excluded from study as failed to amplify
E1497783-N5245720	YCP 10	Feathers	2011	
"Eastern Line"	YCP 11	Whole bird	2011	
	FT3308	Tissue	1996	From Victoria University
Hurunui (from Victoria University)				
	FT3304	Tissue	1996	
	FT3305	Tissue	1996	
	FT3306	Tissue	1996	
	FT3307	Tissue	1996	
	FT3309	Tissue	1996	Excluded from data analysis as 45% missing data (failed to amplify)
	FT3311	Tissue	1996	
Hope Valley Samples (from Victoria University)				
	WG611	Tissue	unknown	
	WG612	Tissue	unknown	

Appendix 2:

Details of samples used in study			
Species	Origin	Specimen ID	Sample / Collection details
C. malherbi	Hawdon	W001	Wing found in Hawdon 2007/2008.
		SB081	Captive bred at Peacock Springs. Dead bird.
		W011	Feathers collected from Hawdon
		SB024	Egg from wild 2004. Hatched at Peacock Springs. Feather sample.
		SB280	Egg from Nest OFP4 2010/2011. Hatched at Peacock Springs. Feather sample.
		W026	Unhatched egg collected from nest OFP15 2010/2011
	Hurunui	SB002	Dead bird. Had been released on Maud Island 2007.
		SB153	Nest OFP2 2007/2008. Feather samples obtained from Peacock Springs
		FT3309	Collected 1996. Blood / tissue obtained from University of Victoria
		FT3314	Collected 1996. Blood / tissue obtained from University of Victoria
		FT3315	Collected 1996. Blood / tissue obtained from University of Victoria
		FT3316	Collected 1996. Blood / tissue obtained from University of Victoria
		FT3317	Collected 1996. Blood / tissue obtained from University of Victoria
	Hawdon x Hurunui	SB120	Dead bird from Peacock Springs.
		SB138	Dead bird from Peacock Springs.
		C001	Dead embryo from Peacock Springs
	Poulter Valley	W003	Embryo collected from nest 2008
		W016	Embryo collected nest OFP7
		SB197	Eggs collected from OFP1 08/09. Hatched at Peacock Springs. Feather sample.
		W007	Unhatched egg collected from nest OFP5.
		W013	Embryo from nest OFP7
		W017	Embryo collected from OFP8.
	Hope Valley	WG611	Blood /tissue obtained from University of Victoria.
		WG612	Blood /tissue obtained from University of Victoria.

Species	Origin	Specimen ID	Sample / Collection details
C. auriceps	Hawdon	YCP 7	Feathers collected from Hawdon 2010
		YCP8	Feathers collected from Hawdon 2010
		YCP10	Feathers collected from Hawdon 2011
		YCP11	Dead bird collected from Hawdon 2011
		FT3308	Collected 1996. Blood / tissue obtained from University of Victoria
	Hurunui	FT3304	Collected 1996. Blood / tissue obtained from University of Victoria
		FT3305	Collected 1996. Blood / tissue obtained from University of Victoria
		FT3306	Collected 1996. Blood / tissue obtained from University of Victoria
		FT3307	Collected 1996. Blood / tissue obtained from University of Victoria
		FT3311	Collected 1996. Blood / tissue obtained from University of Victoria

Appendix 3

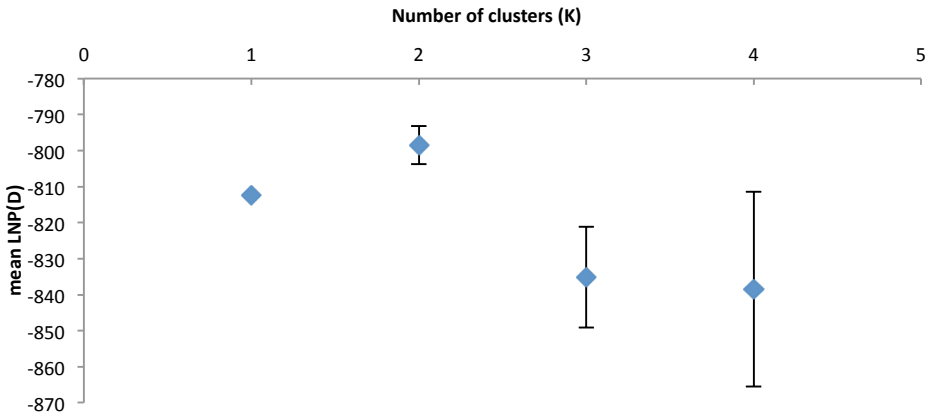

Fig1 : Determination of the most likely number of clusters (K) between all *C. malherbi* & *C. auriceps* samples based on combined microsatellite & RAG-1 data.
Plot of mean (± SD) log likelihoods of STRUCTURE analysis for K = 1 - K= 4

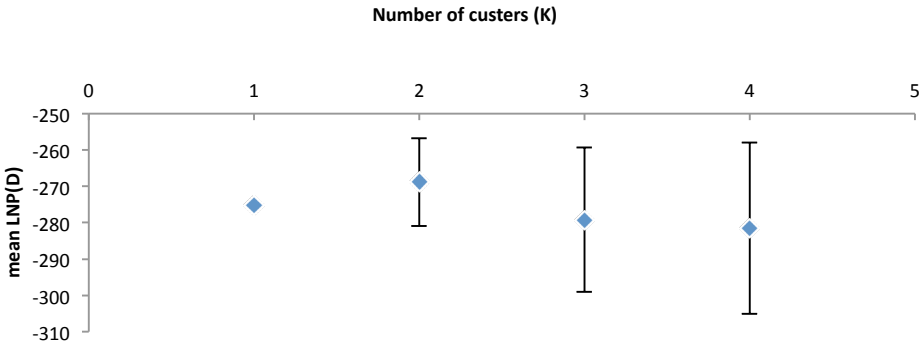

Fig 2: Determination of the most likely number of clusters (K) between all *C. malherbi* & *C. auriceps* samples based on combined microsatellite & RAG-1 data.
Plot of mean (± SD) log likelioods of STRUCTURE analysis for K=1 - K=4

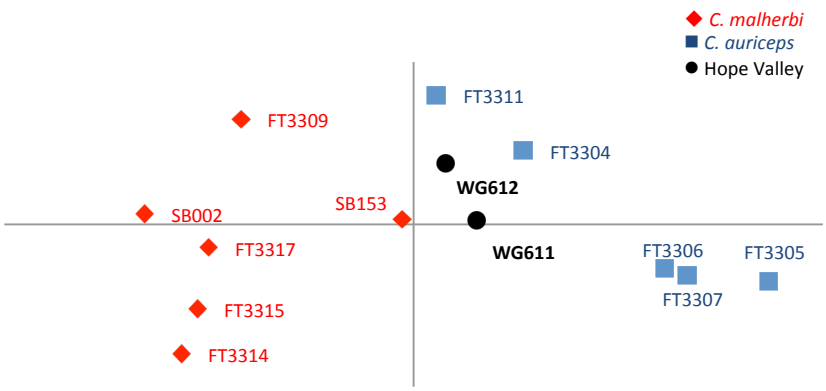

Fig 3: PCA of Hurunui *C. malherbi* & *C. auriceps* samples and Hope Valley individuals based on combined microsatellite & RAG-1 data.